ABSTRACT OF THESIS

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Several aspects of the regulation of cell division in Escherichia coli have been investigated. These include the specificity of septum structure, the determination of the site of cell division, and the interaction between replicons and the process of cell division.

A mutant of E. coli K12 isolated by Dr. H. I. Adler (strain P678-54) was used in the studies on septum structure and the localization of division. The studies on septum structure were carried out in collaboration with Dr. E. W. Goodell and Dr. U. Schwarz of the Friedrich-Miescher-Laboratorium, Max Planck Institute, Tubingen, West Germany. The structure of the septum was found to be very similar to that of the lateral wall. There were, however, two differences: a protein (molecular weight 48,000 daltons) seemed to be specific for the outer membrane of the septum, and a phospholipid, phosphatidyl glycerol, was enriched in the bytoplasmic membrane of the septum.

The localization of division was found to be dependent on two processes in the minicell producing mutant; the formation of a potential division site and its subsequent activation by a factor which behaves as a diffusible entity. This is interpreted to mean that in the wild-type cell there are distinct processes determining the localization and timing of cell division.

The role of a blocked replicon in preventing subsequent cell division was investigated by introducing ultraviolet damaged bacteriophage P1 DNA as a supernumary replicon. When a cell is infected with a damaged P1 genome cell, division is blocked unless the host cell can repair the damaged genome. Replication and segregation of the host cell's chromosome(s) and cell growth are not affected. The kinetics of filament formation suggest that a single blocked replicon is sufficient to block all further cell division. UV survival curves for uninfected cells support this hypothesis.

Use other side if necessary.

The Localization and Timing of Cell Division in *Escherichia coli*.

by

R. M. Teather, B.Sc. (U.B.C.)

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Doctor of Philosophy

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II. The timing of cell division.

Abstract.

Several aspects of the regulation of cell division in Escherichia coli have been investigated. These include the specificity of septum structure, the determination of the site of cell division, and the interaction between replicons and the process of cell division.

A mutant of E. coli K12 (P678-54, isolated by Dr. H. I. Adler) was used in the studies on septum structure and the localization of division. The studies on septum strucure were carried out in collaboration with Dr. E. W. Goodell and Dr. U. Schwarz of the Friedrich-Miescher-Laboratorium, Max Planck Institute, Tubingen, Germany. The strucure of the septum was found to be very similar to that of the lateral wall. There were, however, two differences: a protein (molecular weight about 48,000 Daltons) seemed to be specific to the inner membrane of the septum, and a phospholipid, phosphatidyl glycerol, was enriched in the inner membrane of the septum.

The localization of cell division was found to be dependent on two independent processes in the minicell producing mutant; the formation of a potential division site, and its subsequent activation by a factor which behaves as a diffusible entity. This is interpreted to mean that in the wild-type cell there are also two distinct processes involved in the localization and timing of cell division.

The role of a blocked replicon in preventing subsequent cell division was investigated by introducing ultraviolet-damaged bacteriophage P1 DNA as a supernumary replicon. When a cell is infected with an ultraviolet damaged P1 genome cell division is blocked unless and until the host cell can repair the damaged genome. Replication and segregation of the host cell's chromosome(s) and cell growth are not affected. The kinetics of filament formation suggest that a single blocked replicon is sufficient to block all further cell division. UV survival curves for uninfected cells with different DNA contents support this hypothesis.

I. The Localization of Cell Division.

A. Introduction.

In a rod-shaped organism such as Escherichia coli the site of cell division is normally in the centre of the long axis of the cell. The positional imformation required for this process could in principle be specified in one of two ways. A gradient could be maintained within the cell, extending from two morphologically distinct sources (the cell poles), or a distinct structure associated with the cell envelope could be established and positioned appropriately by localized growth of the cell envelope.

Several types of gradient could be visualized. One possibility is that the altered morphology at the cell pole creates a distortion in the cell envelope, perhaps in a structure such as the protein 'crystal' suggested by Henning, Rehn, and Hohn (1973), which would be transmitted through the envelope (Henning and Schwarz, 1973). Alternatively, a diffusible 'morphogen' might be synthesized at the cell poles, with a concentration gradient being maintained within the cytoplasm (or the membrane) by an enzyme which degrades the morphogen or incorporates it into a polymeric structure. In either of these models, septum formation is initiated at a central minimum. Such hypotheses can also provide a mechanism to control the timing of cell division. If the enzymes responsible for septation are inhibited by the morphogenic structure or substance then a minimum separation of the cell poles would be required before septation could take place.

Another possibility would require that wall synthesis and septation involve a substrate-limited autocatalytic enzyme system, with no localization of either enzyme or substrate (except insofar as both are confined to the cell envelope). In such a case, in a rod-shaped cell, an active enzyme system would tend to form in the center of the cell simply because substrate availability is greatest at that point. In this instance elongation of the cell and septum formation would both take place in the center of the cell and involve a common enzyme system. This model has two features that are attractive. First, it does not require any fixed structures to be located in the cell, and, second, it provides a means of shutting off wall synthesis at the site of septum formation after the completion of septum formation. Such shutoff would be automatic because of substrate deprivation (U. Schwarz, personal communication).

Any alternative to gradient control must involve localized growth of the wall, and hence implies some conservation of cell envelope material which should show a segregation pattern among progeny cells, the specific pattern depending on the specific model being invoked. There is now a reasonable body of evidence to suggest that net wall synthesis occurs at a few restricted sites in a number of bacteria (see section I-E for discussion) so such hypotheses are at least equally likely to be true. The work of Higgins and Shockman (1971; also Higgins, Daneo-Moore, Boothby, and Shockman, 1974) with Streptococcus faecalis provides an example of such a mechanism

In this case, cell wall growth is initiated at an interface between 'old' and 'new' material that can be visualized in the electron microscope as a thickened equatorial ring in a newborn cell. In the case of these spherical cells there is no clear distinction between longitudinal growth and septum formation. New wall material is added on the inside edge of the equatorial ring and split by enzymes to produce the new hemispheres of the two daughter cells.

Rather than discuss in detail the experimental evidence for localized growth of the cell wall in E. coli at this point, I will defer the discussion until Section I-E, where I will present a model for the localization of septum formation. The fact that there is such evidence, however, does not mean that models involving gradient formation are excluded. One potentially useful way of distinguishing between the two types of hypotheses is through the study of mutants in which the localization of division is abnormal. In the next two sections I will describe my work on one such mutant in which division can take place very close to an existing septum, or cell pole.

B. Analysis of the Division Pattern of a Minicell- producing Mutant.

i) Introduction

Escherichia coli K12 strain P678-54, a derivative of strain P678, was originally isolated on the basis of its increased resistance to ionizing radiation (Adler et al, 1966, 1967). This strain shows abnormal localization of septum formation, producing both very small, anucleate 'minicells', by division very close to an existing cell pole, and cells which are much longer than normal. Fig. 1 shows an electron micrograph of a cell of the mutant strain P678-54 which has just completed a polar division to produce a minicell. Similar mutants have been isolated in E. coli by selection for filaments from mutagenized cultures (R. Teather, unpublished). Minicell producing strains have been isolated in many other rod-shaped bacteria, including Erwinia amylovora (Voros and Goodman, 1967), Salmonella typhimurium (Epps and Idziak, 1970; Tankersley, 1970; Tankersley and Woodward, 1973), other species of Salmonella (Epps and Idziak, 1970), Haemophilus influenzae (Setlow et al, 1973), and Bacillus subtilis (Reeve et al, 1973).

In the normal cell cycle of E. coli cell division is the culmination of a series of events which includes a round of chromosome replication and a period of protein synthesis which is normally coincident with the period of chromosome replication. The initiation of these processes is apparently controlled by cell size, with initiation taking place at a constant cell volume / chromosome origin

Fig. 1. Polar division of P678-54. Cells were grown at 37 C in L-broth and prepared for electron microscopy as described in materials and methods. Bar equals 1 um.

ratio (Donachie, 1968). If either chromosome replication (Clark, 1968a; Helmstetter and Pierucci, 1968) or protein synthesis (Pierucci and Helmstetter, 1969) is inhibited, cell division is blocked. Such a block applies also to minicell formation in P678-54 (Clark, 1968a, 1968b; Khatchatourians, 1973). Similarly, mutants defective in normal cell division also appear to be blocked in minicell formation (Adler et al, 1967; Khatchatourians et al, 1973). Thus minicell producing divisions appear to be subject to all normal controls except for the system that specifies the location of the septum.

As noted above, a growing population of strain P678-54 contains abnormally long cells. The range of cell sizes in a normal population of E. coli is only about two-fold, although the average cell size does vary with growth rate. This variation is readily accounted for. Chromosome replication is initiated at a fixed cell volume (Donachie, 1968) and the cell cycle itself, which can be thought of as beginning at the time of initiation of chromosome replication, takes 60 minutes to complete at any growth rate greater than 1 generation / hour (Cooper and Helmstetter, 1968). Thus cells with a generation time of 60 minutes divide at twice the initiation mass; cells with a generation time of 20 minutes divide at eight times the initiation mass. However, these considerations do not affect the relative range of cell sizes in a population at any given growth rate. The ideal of a twofold range in cell size is seldom attained by a bacterial population but the deviation from this ideal is not normally very large. There is some variation in this respect beween different strains of E. coli (Kubitschek, 1969). Such variation as normally

occurs can largely be ascribed to the failure of the daughter cells to separate at a fixed time after the completion of septation. The final stages of cell separation take place 'outside' the cell, and it is perhaps not surprising that the period required to complete this process is rather variable.

In analysing the division pattern of the minicell producing mutant P678-54 I wished to establish how the abnormal size distribution of this strain might arise. In particular, I wished to establish whether there is a correlation between the production of minicells and the length distribution of the culture. It was also of interest to establish the extent to which the location of division sites was still restricted (that is, whether or not the site of cell division is randomly placed in this strain).

ii) Materials and Methods

Strains. *E. coli* K12 strain P678, $thr^- leu^- thi^-$, and the minicell-producing derivative P678-54 (Adler et al, 1967) were used in these experiments. Minicell production has been reported to be the result of two mutations at different loci, one of which is already present in the parental strain P678 and which has no obvious effects on the cell in its own right (Roozen and Curtiss, 1969). The genotype of strain P678-54 is therefore $thr^- leu^- thi^- minA^- minB^-$. Both strains were obtained from Dr. H. Adler.

Media. Cells were grown at 37 C in L-broth, consisting of 10g tryptone (Difco), 5g yeast extract (Difco), and 10g NaCl per liter of distilled water, or in minimal medium, consisting of 007 salts (Clark and Maaloe, 1967) supplemented with 2 mg/ml glucose, 50μg/ml leucine and threonine, and 10μg/ml thiamine. The generation time for both strains was 26 minutes in L-broth and 65 minutes in 007 minimal medium.

Measurement of Cell Length. The distribution of cell lengths in exponentially growing populations was obtained by spreading the cells on thin layers of 1.2% agar containing 0.05% sodium azide and photographing the cells with a Zeiss 'Ultraphot' photomicroscope using phase contrast optics. In some experiments observations were made on living cells growing on thin layers of 1.2% agar containing growth medium. Measurements were made on enlarged projections of the negatives.

Electron Microscopy. Cells were grown in exponential phase to approximately 5×10^7 cells/ml, fixed with glutaraldehyde (Kay, 1965), and centrifuged at 13,000 x g for 30 minutes. This long centrifugation is required to pellet the minicells in the population. The cells were

washed two times by resuspending the pellet in 10 ml of 1% ammonium acetate and repeating the centrifugation. The pellet was then resuspended in 1/20 of the original volume of the culture in 1% ammonium acetate. Samples were placed on freshly prepared grids and stained with uranyl acetate (Kay, 1965). Photographs were taken with a Siemens 'Elmiskop 1A' electron microscope.

Mathematical Procedures. The calculations involved in modelling the mutant population are described in the results section. The basic equations were derived by Dr. J. F. Collins.

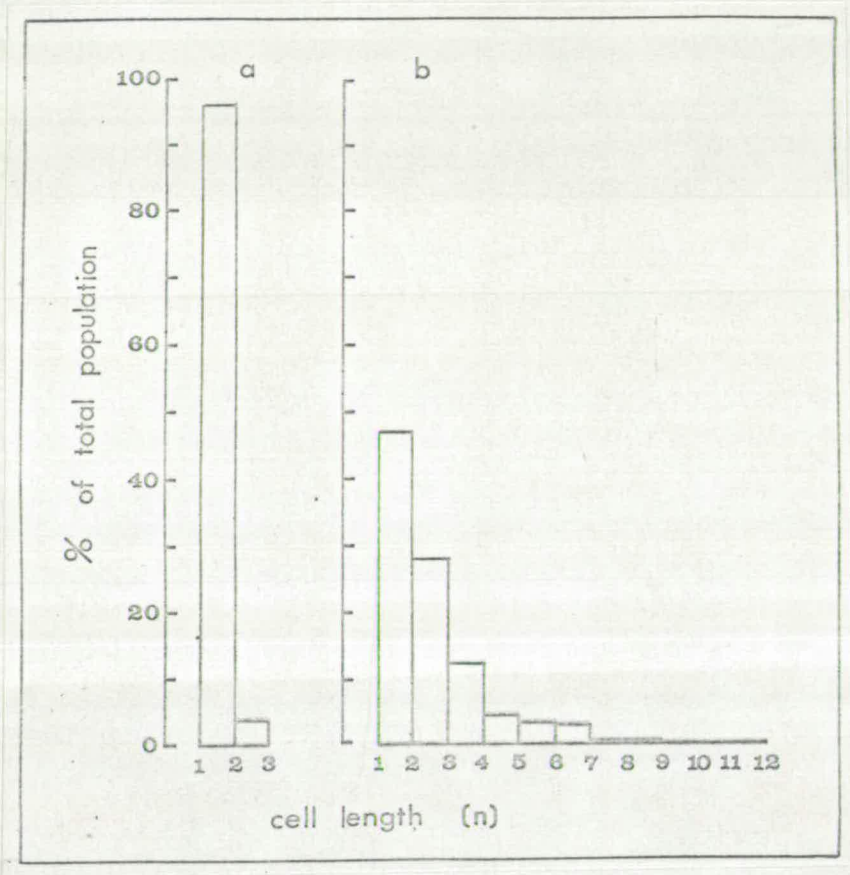
iii) Results

The distribution of cell lengths in an exponential population of the parental strain P678 is shown in Fig. 2a. Over 95% of the cells lie within a twofold range and the distribution therefore corresponds very closely to the size distribution of an 'ideal' population in exponential growth.

The cell length distribution of the minicell-producing strain P678-54 under the same growth conditions is shown in Fig 2b. In this case the range in size is at least ninefold. The minimum length for cells is approximately the same for both the wild-type and mutant populations (excluding minicells from consideration) but the average cell length of strain P678-54 (2.46) is 1.68 times that of P678 (1.46). Cells grown in minimal medium were smaller but the relative range of cell sizes and the shape of the size distribution for P678-54 were the same. Two other minicell producing strains isolated in this laboratory show a similar size distribution to P678-54. (It should be noted that minicell-producing mutants are quite easy to obtain and it does not therefore seem likely that a double mutant is required to express the phenotype.)

A model was devised to account for the length distribution shown in Fig. 2b. The assumptions of the model are as follows. (i) Potential sites for cell division (potential division sites, or PDS) arise during cell growth in the same way in both the wild-type and mutant strains. Such sites are active for only one division in

Fig. 2. Size distributions of strains P678 (a) and P678-54 (b). Cells were grown in L-broth. Cells are grouped in multiples (n) of the minimum length of P678 (L). Sample size was 350 cells (a) and 400 cells (b).



strain P678 but remain active indefinitely in strain P678-54. A PDS at which a division has occurred forms two cell poles. In strain P678-54, subsequent divisions can occur at these sites and will produce minicells. (ii) The probability of division is equal at all PDS in the cell, whether polar or nonpolar. (iii) If a newborn cell in an exponentially growing wild-type population has the length c, then division will not occur until a length of 2c is reached. At this size there is only enough of some specific factor (division factor) for a single division. This division factor is consumed entirely in the formation of a single septum. If there is more than one division site available (as there is in the minicell- producing mutant) the division factor is expressed solely at one of the available sites. This site is chosen at random out of all the available potential division sites.

As a consequence of these assumptions, the length of a newborn cell in a minicell producing strain may be a multiple of the newborn cell size (c) of a wild-type strain. If the length of a particular newborn cell is nc (where n is an integer), the cell after one generation of growth will be 2nc in length and will have produced sufficient division factor to undergo n divisions. The number of division sites available will be the normal number of internal division sites (2n-1) plus the two polar sites, giving 2n+1 PDS.

Using these assumptions it is possible to predict the length distribution of an exponentially growing population of cells. The distribution of cell lengths that will be observed in such a population depends on two characteristics: (i) the distribution of

cell lengths at birth, and (ii) the pattern of length increase during the cell cycle (Collins and Richmond, 1962). The parental strain, P678, shows an almost ideal length distribution for a population in which cells are born at a single length (c) and divide at twice this length. The mean cell size (1.46c) is very close to the theoretical value of 1.44 times the newborn cell size (Powell, 1956).

The model states that in the mutant population an organism of initial length nc will grow to length 2nc and n divisions will then take place. These will be distributed between the 2n-1 internal PDS and the two polar PDS. n+1 progeny will be formed. Septation is possible at either or both poles, but at least n-2 septations must occur at internal PDS, which will result in n-1 progeny each of minimum length c. n+1 units of length remain to be distributed among the n+1 progeny in a random fashion, and all the possible distributions define all the possible ways in which the dividing organism could complete n septations. The probability of any of the progeny receiving x additional lengths of length c is p(x), where

$$p(x)=\frac{(2n-x)!\,(n+1)!\,n!}{(2n+1)!\,(n+1-x)!\,(n-1)!}$$

(Feller, 1950: page 59, equation 5.1). The addition of any units of length c to either of the two potential minicell progeny results in the nonappearance of the minicell and the formation of an additional viable organism in its place. The expectation of progeny of length ac arising from an organism of initial length nc at the end of its growth and division cycle is Pr(n,a) where Pr(n,0)=2p(0) (the probability of producing a minicell) and Pr(n,a)=2p(a)+(n-1)p(a-1) when $a\neq 0$.

Computed values are given in Table I.

Since the progeny from any one size of intial cell vary in length, the distribution of newborn organisms that maintains a stable length distribution as the population grows (an essential feature of a stable exponentially growing population: Collins and Richmond, 1962) is such that the number of progeny of any one size formed after one generation is twice the number of newborn organisms of that size initially present. If the frequency of newborn organisms of length nc is f(n), and of length m(c) is f(m), then

$$\sum_{n=1}^{n=\infty} f(n) \cdot Pr(n,m) = 2f(m)$$

The values of f(n) were computed by simulating the growth of a single organism of length c through many generations until the relative proportions of each size class stabilized. This distribution of newborn cell sizes was converted into the length distribution that should be observed in an exponentially growing population by the equation:

$$Pop(l) = \sum_{n=1}^{n=\infty} f(n) \cdot 2\left(1-2^{(1-b/nc)}\right)$$

where Pop(l) is the population of length less than or equal to l (the sum of the contributions corresponding to each birth size (nc) in proportion to the frequency of such births, f(n)), and the variable b=2nc when 2nc≤l and b=l when 2nc>l. The fraction of the population lying between the lengths l and l+x is calculated as the difference beween Pop(l+x) and Pop(l).(For further discussion see Teather, Collins, and Donachie, 1974: this paper is bound into this thesis as

Table I. Predicted pattern of daughter cell lengths.

Newborn cell size	Properties of the cell at division			Relative frequency of newborn cells of sizes shown[a]								
	Size	No. of division sites available	Division factors available	Mini	1c	2c	3c	4c	5c	6c	7c	8c
nc	2nc	$2n + 1$	n									
1c	2c	3	1	0.67	0.67	0.67						
2c	4c	5	2	0.80	1.00	0.70	0.40	0.10				
3c	6c	7	3	0.86	1.43	0.91	0.51	0.23	0.06			
4c	8c	9	4	0.89	1.89	1.15	0.63	0.30	0.11	0.02		
5c	10c	11	5	0.91	2.36	1.39	0.76	0.37	0.15	0.05	0.01	
6c	12c	13	6	0.92	2.85	1.64	0.88	0.43	0.19	0.07	0.02	
7c	14c	15	7	0.93	3.33	1.89	1.01	0.50	0.22	0.09	0.03	0.01
8c	16c	17	8	0.94	3.82	2.14	1.13	0.56	0.26	0.10	0.04	0.01

a. The expectations given represent the frequency with which parental cells, when they divide, will give rise to daughter cells of the length shown. The sum of the expectations in any given case is equal to n+1, the number of daughter cells produced from one cell. For example, a normal E. coli cell, dividing at length 2c, gives rise to two similar daughter cells of length c. The expectation for cells of length c in this case is therefore 2.

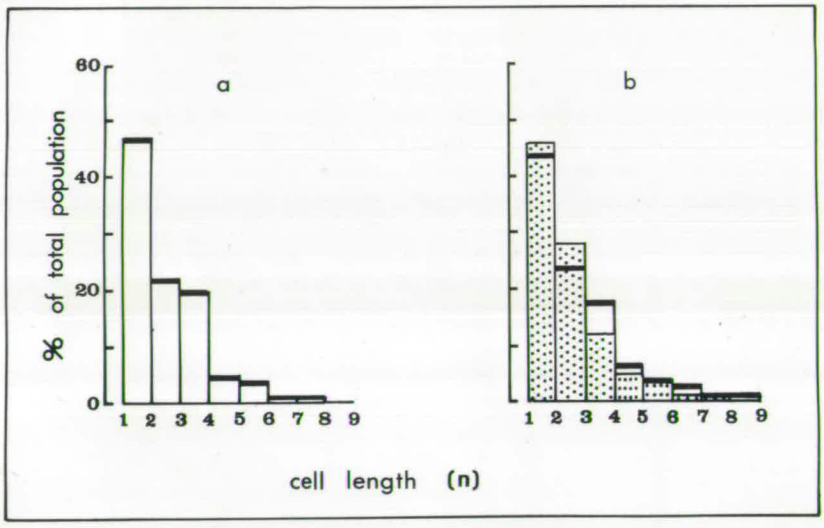
an appendix.)

The length distribution calculated in this way is shown in Fig. 3a. The average length of the population is 2.52c and minicells are produced in the ratio of 0.748 per cell per generation.

Fig.3b shows the same distribution after allowance has been made for classification errors in the assignment of individual cells to particular size classes. This transformation of the theoretical distribution was carried out because there is a significant probability of classification errors in the experimental measurements when the length of the projected image is within 1 mm of a class boundary. Accordingly, 10% of the total number of cells in each size class was transferred to each adjacent size class. Superimposed on this distribution is the experimentally observed distribution. The X^2 value (10 degrees of freedom) was 3.5, indicating a satisfactory measure of agreement ($P < 5\%$ that the distributions are different).

It can be seen from Table I that there is a very high probability that a large cell will give rise to smaller progeny. For example, the probability of a newborn cell of length 8c giving rise to a daughter cell as large or larger is 0.01. In fact, any cell in the population should give rise to a clone with the same size distribution as a normal mutant population within 2 to 3 generations. To test this, individual cells growing on L-broth on slides were observed and photographed at frequent intervals. Cells considerably longer than average were selected for this analysis (at least two times the

Fig. 3. Theoretical size distribution according to the model. (a) predicted distribution. (b) predicted distribution after 'smearing' to allow for probable experimental errors in classification (heavy bar). Superimposed on this distribution is the observed distribution for P678-54 (shaded area).



average length of the population). Fig. 4 shows an example of a life history of such a cell (initial length 5c) and its progeny. Because the model applies only to exponentially growing cells, the rate of growth of such clones was measured. Fig. 5 shows that the growth of the cells under such conditions is in fact exponential. A combined size distribution for the progeny of these large cells after 2-3 generations of growth is shown in Fig. 6, superimposed on the distribution for an exponential population predicted by the model. (The total number of progeny cells measured was 216.) The value of X^2 (10 degrees of freedom) was 3.0, again indicating a satisfactory measure of agreement ($P < 3\%$ that the distributions are different).

Other predictions of the model were tested. An exponentially growing population of wild-type cells ranging in length from c to 2c should have a mean length of 1.44c (Powell, 1956). If the normal minimal newborn cell length of P678-54 is also c under the same growth conditions, then the predicted mean length for P678-54 is 2.52c. The mean lengths observed for P678 and P678-54 were 1.46c and 2.46c, respectively.

Another prediction of the model is that there should be a constant ratio of 0.75 minicells per nucleated cell in an exponential population, as long as the minicells are reasonably stable. A direct count of minicells, using the electron microscope, gave a ratio of 0.67 ± 0.08. This method may possibly underestimate the proportion of minicells; first, because minicells would be preferentially lost during the centrifugation steps involved in preparing the cells for

Fig. 4. Growth and division of a large cell of strain P678-54 growing on L-broth + 1.5% agar (Davis) at 37 C. Arrows show divisions that have taken place since the previous observation. The cells were photographed using phase contrast and measurements were made on enlarged prints from the negatives.

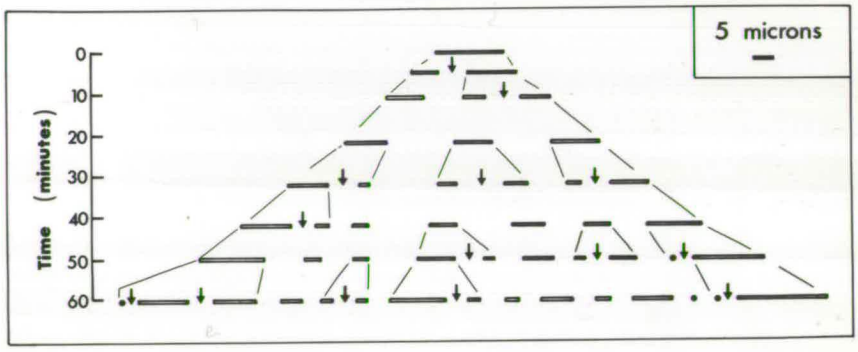


Fig. 5. Growth of P678-54 on L-broth + 1.5% agar (Davis). Each symbol represents one cell (0 time) and its progeny. The log of the sum of the length of all cells in a clone (ordinate) is plotted against time. Measurements were made as described in the legend to Fig. 4. Growth is clearly exponential.

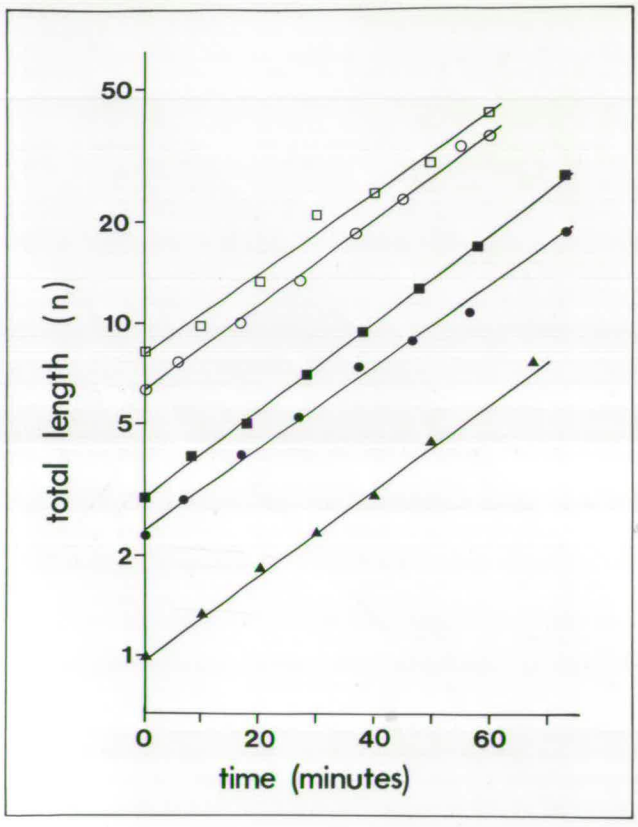
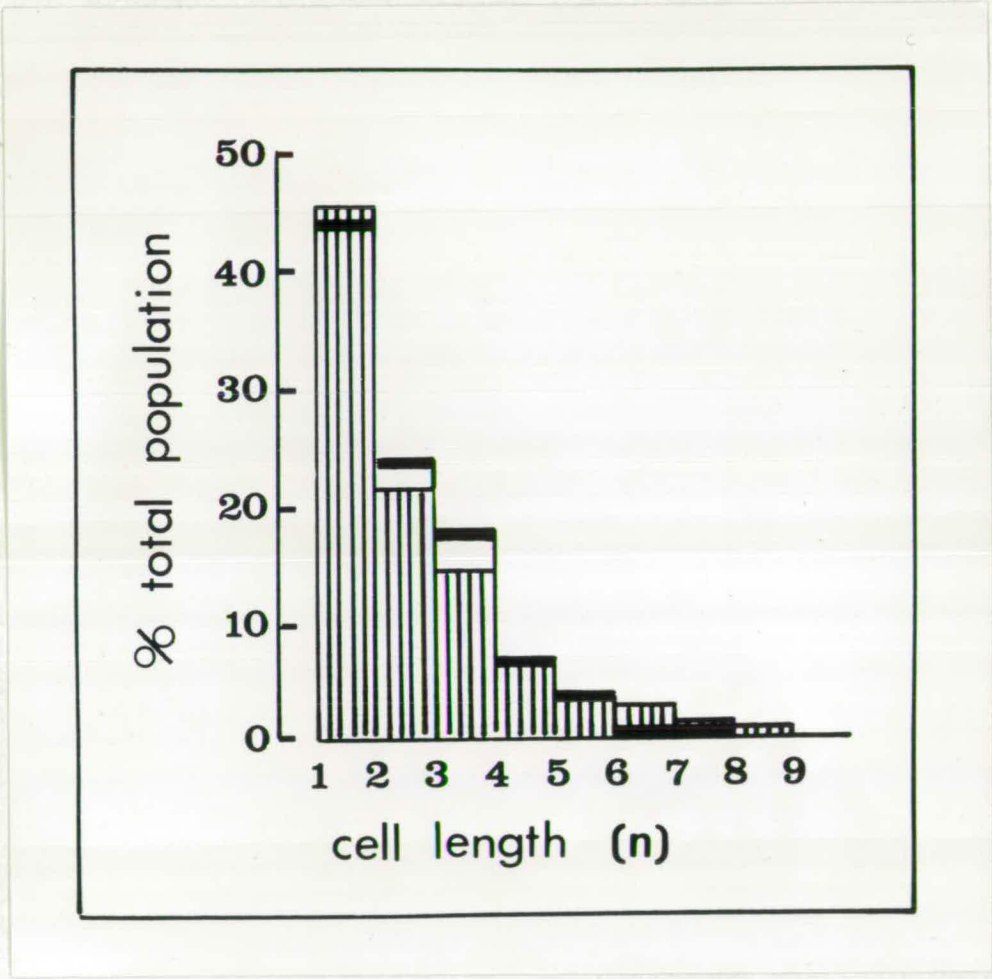


Fig. 6. Size distribution for the progeny of large cells (n>5) of P678-54 after 2-3 generations of growth on L-broth + 1.5% agar (Davis) at 37 C (shaded area). Superimposed on this distribution is the predicted distribution from Fig. 3b. Measurements were made as described in the legend to Fig. 4.



electron microscopy, and second, because the length of time for which the minicells persist after formation is not known. Measurement of the relative frequency of polar and non-polar divisions was therefore made by microscope observation of living cells growing on nutrient agar. The frequency obtained in this way was 0.72 ± 0.11.

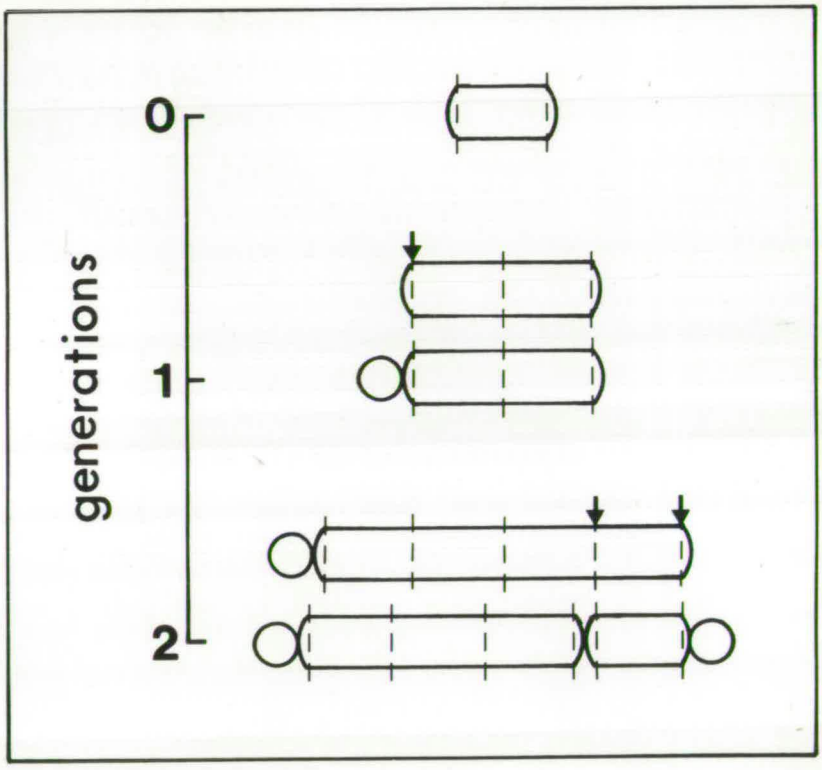
Among cells which divide at a length of 2c, the predicted frequency of polar divisions is 0.67. Direct observation of divisions in cells of this length gave a frequency of 0.61 ± 0.08. Minicells were also observed to be produced with approximately equal frequency at the 'old' and 'new' cell poles (49 divisions were observed at the 'old' pole vs. 43 at the 'new' pole), confirming another assumption of the model.

iv) Discussion.

The above model for the control of cell division in P678-54 provides an accurate description of the mutant's division pattern. Although I have been unable to devise a workable alternative model, it remains possible that one may exist. For example, it has been suggested (Adler and Hardigree, 1972) that the polar division sites of P678-54 are 'immature' division sites that would in the normal course of events be moved to the center of the cell by wall growth before they became available for the initiation of septum formation. (This model would be compatible with the bipolar growth of the cell envelope suggested by Begg and Donachie (1973).) Thus minicell formation would result from the premature activation of these sites. With certain additional conditions such a model could account for the observed distribution of cell sizes. The most unlikely assumption that would be required is that the newly formed PDS, at the cell poles, could be activated only immediately after formation or after one generation of growth. There does not seem to be any reason to make such a hypothesis when a preexisting mature PDS, or at least a part of one, must already be present at the cell poles.

Specific cell histories can be used to show that succeeding cell divisions do not follow a pattern that might be explained by localized wall growth leading to the segregation of one or more active division sites. Fig. 7 shows one such division pattern that has been observed. Thus while the possible locations for septum formation seem to be defined by some measuring process, the activation of these sites is

Fig. 7. One observed division pattern for P678-54 growing on L-broth + 1.5% agar. Dotted lines show the presumed location of potential division sites. Arrows show the sites at which division is initiated.



apparently subject to an independent level of control. The triggering event can be formally described as the production of a diffusible entity (one such entity for the termination of each round of chromosome replication) which activates a single PDS.

Coyne and Mendelson (1974) have shown that in a minicell-producing mutant of Bacillus subtilis the location of polar division sites is not random. However, their studies were performed on germinating spores, following the clones for only 2 to 4 generations after germination. It is unlikely that such cells are in a state of balanced growth. In view of the difficulty of visualizing nascent septa in this organism, and the long and variable period of time that is required to complete cell division even when the cells are in 'balanced' growth, their results cannot preclude the possibility that the mutation in B. subtilis has the same basis that I have ascribed to P678-54.

Two other observations on P678-54 should be mentioned at this point. First, one consequence of the model is that cells with an odd number of nuclei (3,5,7,etc.) should be present in the mutant population because of the random division of the larger cells. Such cells are in fact quite common (W. D. Donachie, personal communication). Secondly, the minimum size for cells with a visible septum is less for cells undergoing a polar division than it is for cells undergoing a normal division (W. D. Donachie, personal communication). This is not a prediction of the model, but it can be explained quite readily. If the signal that activates a PDS is

normally made before the maturation of the PDS, then there would be a lag between the signal and the beginning of septum formation at a new, central, PDS that would not be required if the signal was received by a mature, polar PDS.

The division pattern of P678-54 is not difficult to reconcile with any model for the contol of localization of cell division. If a gradient model is to be compatible with these results, however, certain features of the models normally discussed must be fixed. Once a PDS has been formed it itself must become a source of the morphogen, whether or not it has been used as a division site. This requirement makes the model of Henning and Schwarz (1973) seem unlikely. As a consequence of this requirement, it must be the maturation of the PDS, and not septum formation per se, that is inhibited by the morphogen. Thus this work does not help with the question of how division sites are localized in E. coli, although it does help to show that the localization of division sites and the initiation of septum formation are not part of the expression of a single sequential process. This is conceptually a bit surprising, since any system which specifies a single unique site at which the cell can divide should automatically provide for the synthesis of a single septum. The role for two separate systems in the cell, each of which limits the cell to one division per completed cell cycle, is not clear.

C. A Comparison of the Structure of the Cell Pole and the Lateral Wall.

i). Introduction

Most models for the localization of cell division imply the existence of discontinuities in the cell wall that are recognizable to an enzyme system. In addition to this consideration, the morphology of the cell at the poles (hemispherical) is different from the morphology of the lateral wall (cylindrical). Despite the wealth of evidence that individual components or subunits of the inner and outer membranes and sacculus are freely diffusible, rapidly translocated within the structure, or inserted at random during cell growth (see section I.E for review) there is no reason to believe that local variations in form or subunit composition might not be maintained. An obvious case in point is the maintenance of shape of the cell itself.

There are now indications that at least one of the enzymes involved in synthesizing the mucopeptide component of the septum is unique to this process (Donachie and Begg, 1970; Schwarz, Ryter, Rambach, and Hirota, in preparation) and thus might reasonably be expected to be localized in the cell envelope. Even though septum formation cannot yet be defined in a biochemical sense, at least it is known to be subject to different controls from those regulating the longitudinal growth of the cell envelope. Also, the morphology of the cell is maintained both in isolated murein (Weidel and Pelzer, 1964)

and outer membrane (Henning and Rehn, 1973), in the latter case in the absence of any covalent bonding between the subunits (proteins, phospholipids, and lipopolysaccharides). For all these reasons it seemed worthwhile to to compare in as detailed a manner as possible the composition of the cell pole and the lateral wall.

The minicell producing mutant P678-54 provides a simple means of isolating polar material. If a suspension of cells from a culture of this strain is centrifuged through a sucrose gradient the minicells form a distinct band. At the same time, the longer cells in the culture can also be isolated, so that cell envelope preparations that consist largely (or only) of polar material can be compared with preparations derived from the same culture that contain much less polar material than a normal exponential population. The work on the division pattern of this mutant discussed in the preceding section gives further assurance that although septation in this strain is abnormal in its location, the basic system that determines where division can occur is still functioning.

Shortly after beginning this work, I discovered that other workers had initiated a similar study (E. W. Goodell and U. Schwarz of the Max-Planck-Institut, Tubingen). We decided that we would cooperate on the study, with their group carrying out the work on mucopeptide structure and lipid distribution while I would examine the distribution of membrane proteins. Envelope preparations were made in both laboratories but each preparation was exchanged so that a complete analysis could be carried out. The results of Goodell and

Schwarz are included in this section for the sake of completeness.

ii). Materials and Methods.

Escherichia coli K12 strain P678 and its minicell producing derivative P678-54 were used for this study. The bacteria were grown in L-broth (LB) or in minimal citrate (MC) medium (Vogel and Bonner, 1956) supplementd with 2 g/l casamino acids (Difco) and 2 g/l glucose.

Chemicals. Phosphatidylethanolamine and cardiolipin were kindly supplied by Dr. P. Overath; phosphatidylglycerol was obtained from New England Nuclear, Boston, U. S. A. 2-keto-3-deoxyoctonate (KDO) was kindly supplied by Dr. O. Luderitz. All other chemicals were obtained from normal commercial sources.

Separation of minicells and filaments. Cultures were harvested at a density of 1-2 x 10^8 cells/ml in a sorval RC2B or a Sharples continuous flow centrifuge, depending on the volume of the culture.

For smaller cultures (up to 3 l), the culture was centrifuged at 15,000 x g for 15 min at 4C . The pellet was resuspended in cold growth medium (30 ml/l of the original culture medium) and centrifuged at 2,000 x g for 10 min. This centrifugation was repeated twice: the final pellet contained primarily large cells and the three supernatants primarily minicells. The minicells were pelleted by centrifugation at 15,000 x g for 10 min. The pellets were resuspended in a small volume (10 ml/l of the original culture) of 5% sucrose (w/v) in 10 mM Tris HCl, pH 7.8, and 2.5 ml was layered on a step gradient consisting of 6 ml each of 10, 20, 30, 40, and 60% sucrose in the same buffer. In most cases some cell lysis occurred in the

filament fraction and it was necessary to add DNAse (0.1 mg/ml, deoxyribonucleate oligonucleotide-hydrolase (E. C. No. 3.1.4.5) from beef pancreas, obtained from Sigma London) to this fraction before layering it on the sucrose gradient. The gradients were centrifuged at 3,000 x g for 10 min and the bands containing filaments (40% sucrose) and minicells (10% sucrose) were withdrawn using a large bore needle and syringe. The minicells were further purified by passage through a 3 um Millipore filter (SSWP 14200), largely to remove cell ghosts which also band in the 10% sucrose.

Larger cultures (up to 40 liters) were harvested using a continuous flow Sharples centrifuge with a cellophane liner. The flow rate was adjusted so that at least 80% of the minicells were retained in the centrifuge. After the centrifugation the liner was removed and the pellet was divided into two fractions; the upper third of the pellet was greatly enriched for minicells, the lower portion of the pellet (near the inlet) was enriched for filaments. The minicell-enriched fraction and about 1/5 of the filament enriched fraction were resuspended in 5% sucrose (w/v) in 1 mM Tris HCl, pH 7.8, and further purified using sucrose gradients as described above.

Membrane preparation. The sucrose concentration in the purified minicell and filament fractions was adjusted to 0.75 M with 60% sucrose (w/v) in Tris HCl, pH 7.8. Inner and outer membrane fractions were prepared according to the technique of Osborn, Gander, Paris, and Carson (1972) except that with the minicell fraction it was necessary to increase the concentration of lysozyme to 0.5 mg/ml to obtain efficient cell lysis.

Polyacrylamide gel electrophoresis. The membrane pellets were resuspended in sample buffer, consisting of 20% glycerol (v/v), 5% mercaptoethanol (v/v), 3% sodium dodecyl sulphate (w/v), and 0.0625M Tris HCl, pH 6.8 (Laemmli, 1970), to give a protein concentration of approximately 3 mg/ml. The membranes were solubilized by heating to 90C for 4 min. The gel buffer system used was that of Laemmli (1970). The running gel (17 cm in length) contained 10% acrylamide and 0.27% bisacrylamide. The stacking gel (1 cm in length) contained 3% acrylamide and 0.08% bisacrylamide. Electrophoresis was at 0.3 mA/gel (stacking) and 0.8mA/gel (running). Total electrophoresis time was approximately 12 hours. After electrophoresis the gels were fixed in 20% sulphosalicylic acid, stained in 0.1% Coomassie Brilliant Blue (CBB) in 12.5% trichloroacetic acid (TCA) for 6 hours, and destained in 10% TCA, usually overnight. After the gels had been scanned at 585 nm with a Vitatron TLD100 densitometer (Dieren, Holland) they were sliced longitudinally with the apparatus designed by Fairbanks, Levinthal, and Reader (1965) and dried onto Whatmann 3MM filter paper. The apparatus used to dry the gels consisted of a perspex sheet, 30 cm x 30 cm x 1.2 cm thick, with a tubing connector fitted to a central hole, to serve as a platform. Over this was laid a piece of fine stainless steel gauze (19 cm x 19 cm, ca. 50 strands/inch) to serve as a porous layer and also to provide mechanical support over the central hole. On top of this was laid the filter paper (19 cm x 19 cm), the gel (ca. 16 cm x 11 cm), and a piece of Saran Wrap sufficiently large to cover the filter paper. Finally, a square of 1.5 mm thick neoprene rubber sheet with the same dimensions as the perspex base was laid on top so that it overlapped the other components on all

sides. A vacuum was applied via the tubing connector and the entire sandwich was heated with a heat lamp. The Saran Wrap serves to prevent the filter paper and gel from sticking to the hot rubber. Drying was complete in about 1 hour. Autoradiographs were made using Kodak 'Kodirex' x-ray film.

Phospholipid analysis. Cultures were grown for at least four generations in 150 ml MC medium containing 0.01 mM (2-3H) glycerol (380 uCi per umole). The minicells and filaments were isolated and 100 mg (wet weight) of unlabelled cells was added to both preparations. The inner and outer membranes were isolated and phospholipids extracted according to Osborn et al. (1972). The phospholipids were separated by thin-layer chromatography on Silica Gel 60 (merck) using a chloroform:methanol: glacial acetic acid (65:25:8, v/v) solvent system. The position of the individual phospholipids was visualized by exposing the plates to iodine vapour. The appropriate areas of the gel were scraped off and radioactivity was counted in 5 ml of triton-toluene scintillation fluid (10 g PPO in a mixture of 1 l toluene and 0.5 l triton x-100).

Murein analysis. Cells were grown in 500 ml MC medium containing 3.3 uM (3H) meso-2,6-diaminopimelic acid (300 uCi per umole, generally labelled). Minicells and filaments were isolated and sacculi prepared according to Schwarz and Leutgeb (1971).The purified sacculi were digested with lysozyme and the digestion products were separated by descending paper chromatography as described by Leutgeb and Schwarz (1967). The paper (2043 bMgl) was obtained from Schleicher and Schull, Dassel, W. Germany.

Enzyme assays. NADH oxidase and succinate dehydrogenase were measured

as described by Osborn etal. (1972).

Protein determination. Protein concentration was determined by the method of Lowry etal. (1951) using egg white lysozyme as the standard.

KDO determination. KDO was determined as described by Osborn etal. (1972).

iii). Results

Preparation of inner and outer membrane from minicells and filaments. Typical minicell preparations contained 10^3 to 10^4 minicells per nucleated cell. Filament preparations consisted of nucleated cells with an average length 3 to 4 times that of an exponential culture of the wild type strain P678, and contained less than 1 minicell / 100 nucleated cells.

Membrane preparations, after isopycnic sucrose gradient centrifugation, showed considerable variation in the proportion of H band (outer membrane), M band (unfractionated outer and inner membrane), and L1 and L2 bands (inner membrane). There was, however, no consistent difference between minicells and filaments. Fig. 8 shows a typical gradient. The distribution of several enzymes that are known to be located in the cytoplasmic membrane and of 2 keto-3-deoxyoctonate (KDO) was measured to establish the purity of the inner and outer membrane fractions (Table II). KDO is part of the lipopolysaccharide complex of the outer membrane (Heath and Ghalambor, 1963). These data indicate that the outer membrane of the minicells is somewhat less pure than that derived from filaments. This difference may be associated with the difficulty we encountered in achieving efficient lysis of the minicells. The inner membrane preparations show a considerable amount of contamination with outer membrane material (20-30%), but there was no difference in this respect between minicells and filaments.

Fig. 8. Fractionation of E. coli membranes by sucrose gradient centrifugation. A culture (12 l) was grown to a titre of 10^8 cells/ml in minimal medium (MC). Filaments and minicells were isolated. Outer and inner membranes were prepared from both cell types. They were separated by sucrose gradient centrifugation. The figure shows the sucrose gradient of the minicell preparation. Open squares are protein (ug/ml); circles are succinate dehydrogenase (SDH) activity (nmol/min/fraction).

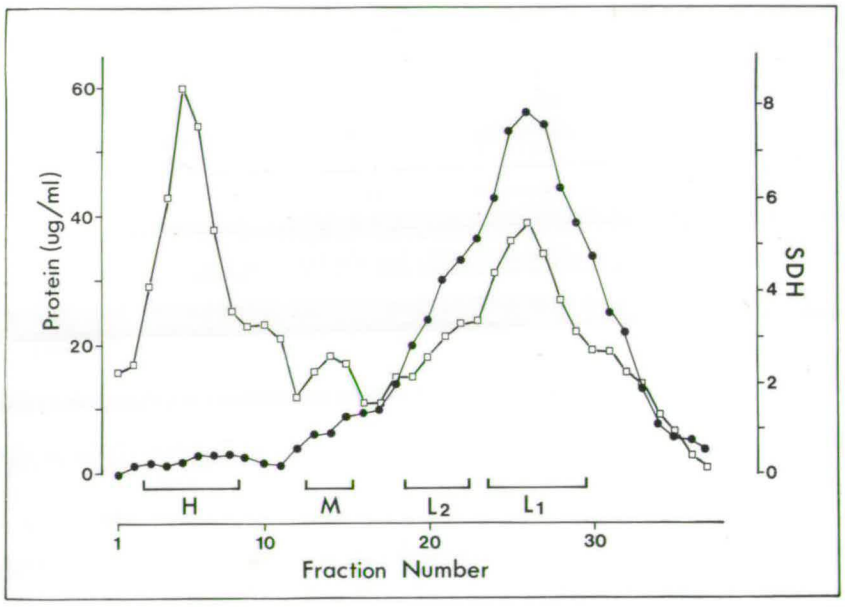


Table II. Enzyme activities and concentration of 2-keto-3-deoxyoctonate in membranes isolated from minicells and filaments.*

	minicells		filaments	
	L1	H	L1	H
NADH oxidase	1900	8	2300	8
succinate dehydrogenase	180	4.2	200	2.5
2-keto-3-deoxyoctonate	38	106	60	150

*Specific activities of the enzymes are given in nmoles per min per mg of membrane protein; concentration of KDO is given in ug per mg of membrane protein.

Membrane composition. The protein composition of membrane preparations was determined by SDS-polyacrylamide gel electrophoresis. The composition of both outer and inner membrane derived from minicells was very similar to that from filaments. However there was one protein in the cytoplasmic membrane which was enriched in the minicell preparations. Fig. 9 shows scans obtained from gels stained with Coomassie Brilliant Blue (CBB). Pattern a. is a scan of the minicell inner membrane preparation; pattern b. is that of the filament preparation. The two dashed lines indicate the position of the peaks on either side of the peak of interest, which is very much reduced in the filament preparations relative to the minicell preparations. These membranes were obtained from cells grown in complete medium (LB). Fig. 10 demonstrates that the same difference is found if the cells are grown in minimal medium (MC). Patterns a. and b. show, respectively, the upper third of the profiles obtained from a single gel containing unlabelled minicell inner membrane (99% of the total protein) and 14C-amino acid labelled filament inner membrane. The gels were stained with CBB to measure the minicell proteins; the radioactively labelled filament proteins were measured by autoradiography. It is apparent that the same peak (between the dashed lines) is absent in the filament profile. Pattern c. shows the relevant portion of an autoradiograph obtained from 14C-amino acid labelled minicells. A comparison of Figs. 9 and 10 will show that while there can be considerable variation in relative peak sizes and in the presence of certain bands between different preparations (particularly with cells grown in different media), most of the major peaks are readily identifiable. The minicell-specific peak was

Fig. 9. SDS-polyacrylamide gel profiles of inner membrane protein from minicells and from filaments. Gels were stained with Coomassie Brilliant Blue and optical density was measured at 585 nm: (a) minicell inner membrane, (b) filament inner membrane. The cells were grown in LB to a density of 10^8 cells/ml.

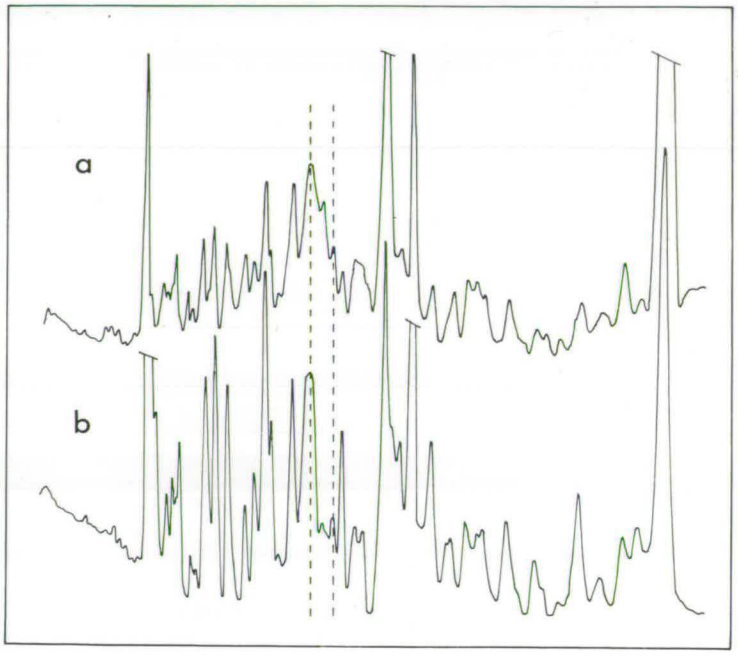
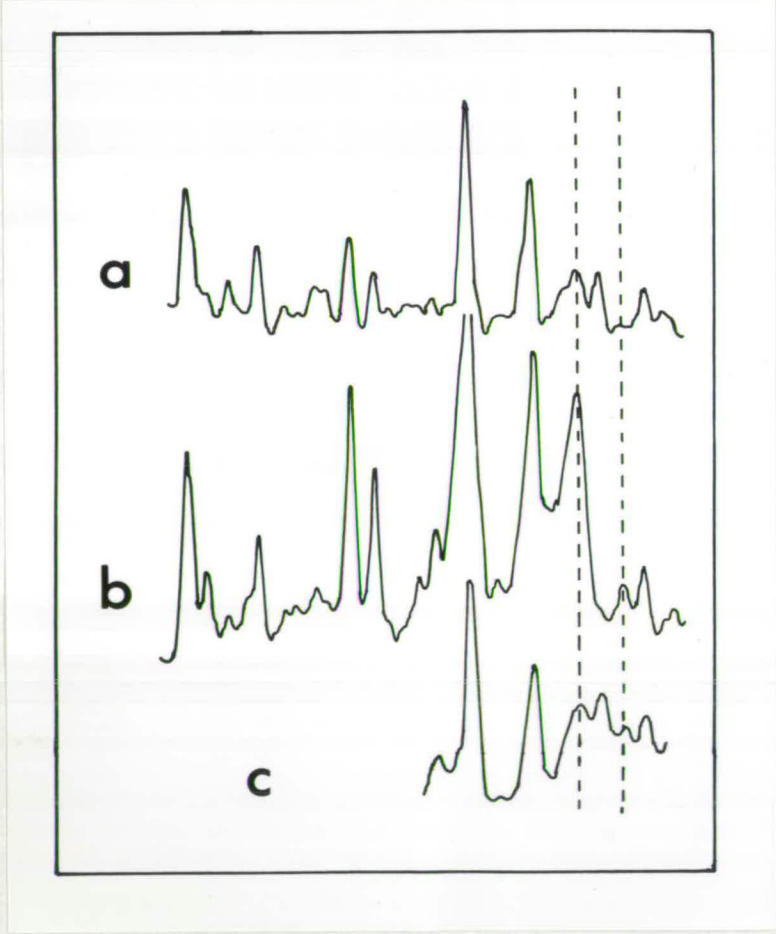


Fig. 10. SDS-polyacrylamide gel profiles of inner membrane protein from cells grown in minimal medium (MC). P678-54 was grown for five generations in 20 ml minimal medium (MC) containing 250 uCi (14C) amino acids (0.18 Ci per atom, generally labelled) and in 15 l unlabelled MC medium to a titer of 10^8 cells/ml. Minicells and filaments were prepared from both cultures. Unlabelled minicells were then mixed with labelled filaments and unlabelled filaments mixed with labelled minicells such that 99% of the protein came from unlabelled cells. Inner membrane was isolated from the two samples; the inner membranes were removed from the sucrose gradient, washed once in Tris.HCl (PH 7.8), and rebanded on a second sucrose gradient. A and B are the two profiles from the gel containing unlabelled minicell inner membrane (a) and labelled filament inner membrane (b) proteins. The gel was stained with CBB to measure the minicell proteins; an autoradiograph was made of the gel to measure the filament proteins. Only the upper third of the profiles is shown. C is from an autoradiograph of the gel containing labelled minicell inner membrane and unlabelled filament inner membrane.



consistently found in all of our preparations; it has a molecular weight of approximately 48,000 daltons.

Fig. 11 shows a typical stained profile obtained from outer membrane preparations. These preparations were very inconsistent in the amount of the first major peak (arrow) present, as well as showing considerable variation in the peaks in the region under the bracket. There were no consistent differences between minicell and filament preparations. Peak 1 (89,000 daltons) was often found to be higher in minicell preparations but the results were not entirely consistent. This peak corresponds in molecular weight to the first peak in Figs. 9 and 10 and might be a contamination from the inner membrane.

The phospholipid composition of minicell and filament membranes was also compared. Unexpectedly, it was found that the phosphatidylglycerol content of minicell membranes, especially the inner membrane, was significantly higher than that of the filaments (Table III). It is also higher than usually found for membranes isolated from other strains of E. coli (Ohki, 1972; White, Lennarz, and Schnaitman, 1972) and from Salmonella (Osborn et al, 1972).

Sacculus. The sacculus is sandwiched between the inner and the outer membrane and gives the envelope its mechanical stability. It is composed of murein, a covalently linked net of polysaccharide chains and short peptide chains (for references see Rogers, 1970; Braun, Gnirke, Henning, and Rehn, 1973). At present it is possible to measure two basic parameters of E. coli murein: the length of the

Fig. 11. SDS-polyacrylamide gel profile of the proteins from the minicell outer membrane. The gel was stained with CBB and scanned at 585 nm.

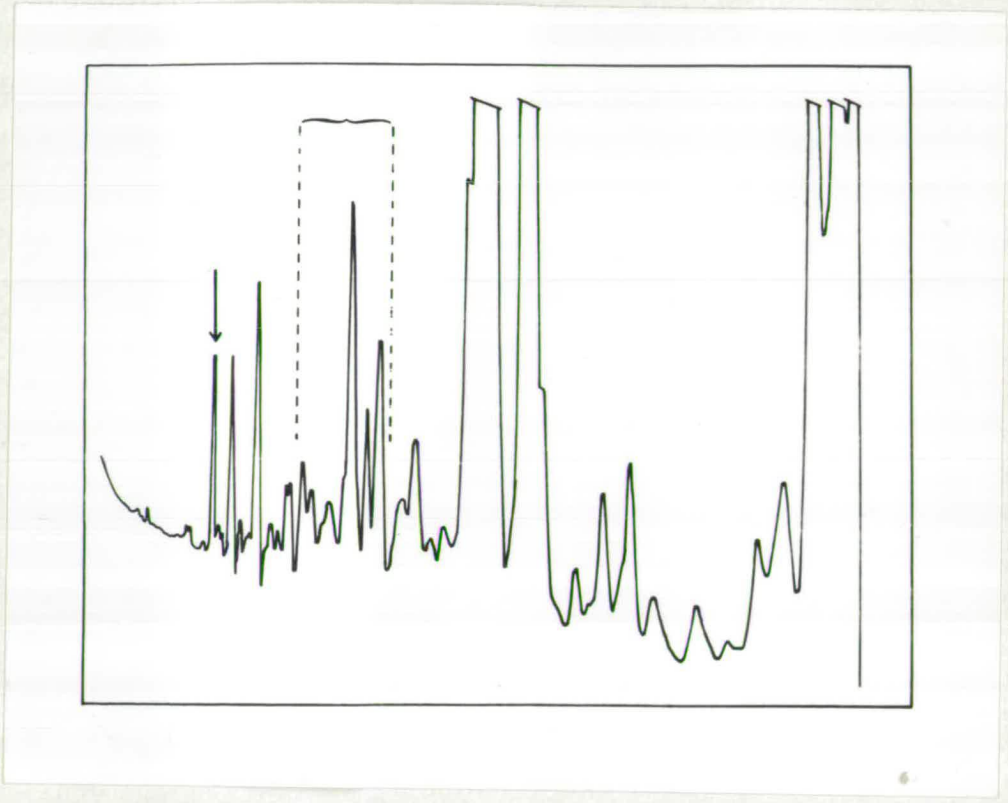


Table III. Phospholipid composition of of filament membranes.*

		Percent (3H) glycerol recovered in: Phosphatidyl-ethanolamine	Phosphatidyl-glycerol	Cardio-lipin
Minicell	L1	37,46	44,52	19,5
	H	66,68	18,26	16,6
Filament	L1	49,57	30,32	20,12
	H	78,73	13,19	10,8

*Membrane fractions were prepared from minicells and filaments which had been grown in the presence of (2-3H) glycerol and the phospholipids were isolated and fractionated as described in Materials and Methods. The data are from two separate preparations. The difference in cardiolipin values between the two preparations is due to the stage in the growth cycle at which the cultures were harvested.

polysaccharide chains and the proportion of completed peptide cross-links. In minimal medium (MC), approximately 50% of the peptide chains are normally cross-linked (Schwarz and Leutgeb, 1971). Minicells and filaments both have 50% of their peptide chains cross-linked (Table IV). Van de Loo and Henning (personal communication) have found that the length of polysaccharide chains from minicells and filaments is nearly identical. The murein of the cell poles of Bacillus subtilis has been shown to differ from the lateral wall in that it is less sensitive to autolytic enzymes (Fan, Pelvit, and Cunningham, 1972; Fan, Beckman, and Beckman, 1974). Thus the situation in E. coli and B. subtilis may well be different.

Table IV. Percent of crosslinked and unlinked peptides in minicell and filament murein.*

	Percent (3H)diaminopimelic acid recovered in:	
	C3+C4	C5+C6
minicells	55	44
	54	45
filaments	54	45
	58	41

*Murein was labelled with (3H)diaminopimelic acid. The murein was isolated, digested with lysozyme, and the digestion products separated by paper chromatography as described in Materials and Methods. Lysozyme degrades the polysaccharide chains to the B-1,4-N-acetyl-glucosamyl- N-acetylmuramic acid disaccharide. The peptide chains are left intact by lysozyme treatment; they are covalently bound to the muramic acid units. The percent of C3 and C4 is a direct measure of the percent of cross-linked peptide chains. C6 and C5 are single disaccharide chains in which the peptide chain is not crosslinked. The data are from two separate experiments.

iv). Discussion

The results of this study indicate that the composition of the cell envelope at the cell poles is very similar to that of the lateral wall. I found, however, one protein which was reproducibly enriched in minicell cytoplasmic membrane. The lipid composition of membranes from minicells and filaments, especially the cytoplasmic membrane, was also different; membranes isolated from minicells had relatively more phosphatidyl glycerol and less phosphatidyl ethanolamine. The role which these differences in protein and lipid composition play in the structure or formation of the cell poles is a matter of conjecture. They may cause the envelope to assume an altered shape; another possibility is that the minicell-specific protein is an enzyme which is involved in the synthesis of the septum. The protein accounts for only about 0.5% of the total minicell envelope protein, and could thus not be considered a major structural component.

Most studies of biological membranes have indicated that proteins and lipids are free to diffuse within the plane of the membrane (Van Tubergen and Setlow, 1961; Beachey and Cole, 1967; Overath, Schairer, and Stoffel, 1970; Overath, 1971; for other references see Singer and Nicholson, 1972). Nevertheless, our results now suggest that local variations in the lipid and protein composition of the membrane do exist. It has, however, previously been shown that local variations in enzyme activity within the cell envelope can occur; during division, E. coli cells have a sharp growth zone at the site of the developing septum where all of the new murein is incorporated into the sacculus

(Schwarz, Asmus, and Frank, 1969; Hoffman, Messer, and Schwarz, 1972; Ryter, Hirota, and Schwarz, 1973). There have been a number of other reports that suggest that certain components of the cell envelope cannot diffuse freely. Autissier, Jaffe, and Kepes (1971) have shown that certain transport proteins, presumably associated with the cytoplasmic membrane, are segregated in large blocks among daughter cells. Green and Schaechter (1972) have presented evidence which suggests that phospholipids in E. coli are not freely diffusible but segregate as relatively large blocks of membrane material among the daughter cells. Begg and Donachie (1973, and personal communication) and Leal and Marcovich (1971) have demonstrated that phage T6 receptors, which are associated with the outer membrane, are segregated in large blocks on the cell surface and are thus also not free to diffuse in the plane of the membrane. Finally, Beachey and Cole (1966) have shown using immunofluorescence that some polar antigens on the cell surface are not diluted by cell growth, at least when complexed with antibodies.

The increased level of phosphatidylglycerol in the minicell could conceivably be the result of the isolation of the minicell from the rest of the cell at a particular time in the cell cycle rather than a reflection on the mobility of phospholipids in the cell membrane. It is known that phosphatidyl glycerol has a rapid rate of turnover in growing E. coli cells and that this rate varies during the cell life cycle (Ohki, 1972; Ballesta and Schaechter, 1971); it is possible that local or cyclic variations in this turnover rate lead to the increased concentration of phosphatidyl glycerol observed in minicells.

D. Time of Synthesis of Septum Components.

i) Introduction.

When either DNA replication (Clark, 1968; Helmstetter and Pierucci, 1968), RNA synthesis (Jones and Donachie, 1973), or protein synthesis (Pierucci and Helmstetter, 1969) is inhibited in an exponentially growing culture of E. coli, cell division stops. However, there is a period of residual division which lasts about 20 minutes at 37 C. The fraction of the population that divides in this time is related to the generation time of the culture. It has been shown that the cells which undergo this 'residual' division are those cells in which a round of chromosome replication has been completed within the 20 minutes preceding the block (Clark,1968; Cooper and Helmstetter, 1968). The processes of the cycle that follow the termination of a round of chromosome replication require about 20 minutes to complete (at 37 C) at all growth rates greater than 1 generation/hour. This part of the cell cycle is termed the 'D' period (Cooper and Helmstetter, 1968), and once a cell has entered the D period it can complete the cell cycle (ie., divide to produce two daughter cells) in the absence of DNA, RNA, or protein synthesis.

Since the cell can synthesize a septum in the absence of protein synthesis any enzymes specifically required for this process must be present in the cell before they are actually used. In addition, the protein components of the septum must somehow be provided. There are a

number of ways in which this might be accomplished. The proteins might be present as a pool within the cytoplasm of the cell, they might be derived from existing membrane, or they might in fact not be necessary for membrane synthesis at all, in that it might be possible to increase the area of the membranes by inserting lipids without adding any protein molecules. This last possibility seems rather unlikely in the case of the outer membrane since it normally contains enough protein to form a continuous protein 'crystal' and is mechanically stable in isolation from the rest of the cell wall (Henning et al, 1973). This suggests that protein-protein interactions must play a role in maintaining the integrity of this layer of the wall and that some protein would therfore be required to synthesize this part of the septum. There is one other possibility, which is that there is no net growth of the cell wall during septation: the septum could be derived from existing cell wall by rearrangement without decreasing the length of the cell(s), although the cell volume would be somewhat reduced.

These alternatives might be tested in several ways. For example, one could label the proteins of the cell wall for one generation of growth with radioactive amino acids and then block protein synthesis. The specific activity of the protein in the cell wall at the end of the labelling period should then be 0.5 (relative to the maximum possible specific activity attainable under the labelling conditions being used), while any pool of septum-specific proteins in the cytoplasm would be expected to have been synthesized within the labelling period and hence would have a specific activity of 1.0. As these proteins are inserted into the wall to form the septum the

specific activity of the cell wall in the culture as a whole should increase.

The increment in specific activity which could be obtained depends on the growth rate of the culture, which determines both the fraction of the culture which will divide when protein synthesis is inhibited and the relative surface areas of the septum and the lateral wall. (The increase in cell volume that occurs with increased growth rate is to a large extent accomplished through an increase in the diameter of the cell, which means that at higher growth rates the area of the cell poles is relatively greater compared with the area of the lateral wall.) The greatest possible increment would be obtained with a culture growing in nutrient broth (generation time ca. 20 min), in which case all of the cells in the culture would be expected to divide once and a cell pole accounts for about 1/5 of the surface area of the cell. Unfortunately, it is not practical to use labelled amino acids in such a growth medium. In minimal medium (generation time ca. 60 min) less than 1/3 of the population would be expected to divide and the cell pole accounts for only about 1/7 of the surface area of the cell. The increment in specific activity that could be obtained in this case, even if there was a septum-specific pool of proteins in the cell at a specific activity of 1.0, would be difficult to demonstrate.

An alternative approach would be to synchronize a culture so that even in minimal medium all of the cells would divide in the absence of protein synthesis. A single synchronous round of DNA replication and cell division can be induced by first blocking protein synthesis,

which allows rounds of chromosome replication in progress to terminate (Maaloe and Hanawalt, 1961; Hanawalt, Maaloe, Cummings, and Schaechter, 1961; Lark, Repco, and Hoffman, 1963; Abe and Tomizawa, 1967; Bird and Lark, 1970) (or nearly so: according to Marunouchi and Messer (1973) protein synthesis is necessary to allow termination of chromosome replication) and cells in the D period to divide, and then blocking further DNA replication but allowing protein synthesis to resume, which allows the cells which have terminated chromosome replication in the absence of protein synthesis to divide and prepares all of the cells in the population to initiate a new round of chromosome replication(Donachie, Hobbs, and Masters, 1968; Donachie, 1969). When protein synthesis is allowed to resume there is a single synchronous round of chromosome replication and cell division (Jones and Donachie, 1973).

ii). Materials and Methods.

E. coli K12 strain W3110, $thy^{-}su^{-}lacY14amb^{-}str^{r}ilv^{-}$, obtained from Dr. M. Monk, was used in these experiments. It was grown in 007 medium at 37 C. In addition to thymine (40 ug/ml), isoleucine and valine (50 ug/ml), and glucose (2 mg/ml) the medium was supplemented with the following amino acids (at the noted concentration in ug/ml): alanine (10), arginine (6.5), aspartic acid (9), glutamic acid (12.5), glycine (5), leucine (12), lysine (5.5), phenylalanine (7), proline (6), serine (5), threonine (6), and tyrosine (13.5). Labelling of membrane proteins was carried out using an equimolar mixture of 14C-labelled amino acids (Radiochemical Center, Amersham: code CFB.152) corresponding to the amino acid supplements listed above. The object was to label all of the proteins of the cell wall to the same specific activity so that stained and autoradiographed profiles of polyacrylamide gels would be directly comparable.

The synchronization procedure used was that of Jones and Donachie (1973), except that amino acid starvation rather than rifampicin was used to block protein synthesis. Medium changes were carried out when necessary by filtration of the culture onto a Millipore filter (0.45 um pore size, 47 or 142 mm diameter depending on the volume and cell density of the culture) followed by washing with prewarmed 007 medium. The cells were resuspended by shaking the filter in the new, prewarmed medium and the filter was immediately removed.

Procedures for membrane isolation and gel electrophoresis are the same as described in section I.C except that in the experiment involving ^{14}C-labelling the inner and outer membranes were not purified, the crude membrane preparations being examined instead. This was done because of the small amount of material available. Gel electrophoresis was carried out in a slab gel apparatus (Studier, 1973) rather than in tubes. Gels were stained and prepared for autoradiography as described in section I.C.

Radioactivity was counted by precipitating samples onto filter paper discs (Whatmann 3MM, 24 mm) and processing them as described by Bollum (1968), except that the ethanol-ether and diethyl ether washes were replaced by washing twice in 95% ethanol. The papers were placed in scintillation counter vials in 5 ml of Butyl-PBD-toluene scintillant (5 g of 2 - (4-tert-butylphenyl) -5- (4-biphenyl) -1,3,4-oxadiazole (CIBA) in 1 l toluene) and radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

iii). Results.

Fig. 12 shows the growth of a culture during and after the pretreatment and labelling periods. The rate of incorporation of the label approaches its maximum value in a very short period of time, so that if the level of 14C incorporated by the end of the labelling period is added to all of the earlier values the points fall on a straight line (on a semilogarithmic plot) that is parallel to the rate of mass increase of the culture (inverted triangles). At the growth rate of this culture (ca. 60 min generation time) most of the cells of the culture should divide once during the pretreatment, and this is the case (residual division is about 87%).

Fig. 13 shows the protein composition of the crude membrane preparations, made from samples taken at the times indicated in Fig. 12. There are striking changes in these patterns with time, in particular in the peaks around 65,000 Daltons in molecular weight shortly after the addition of thymine (sample 3), but there is very little change over the course of the pretreatment (samples 1 to 2). Fig. 14 shows autoradiograms prepared from the same gels. None of the changes apparent in Fig. 13 occur, and if there is any incorporation of previously synthesized membrane proteins after the labelling period it is not into any specific peak(s) but must be occuring equally for all of the membrane proteins. The specific activity of these proteins (Table V) is the same as the specific activity of the culture as a whole, and goes down at the rate that would be expected if incorporation of labelled material into the wall stopped as soon as

Fig. 12. Growth of the culture during and after the pretreatment. An exponential culture of E. coli K12 strain W3110 was grown in 007 medium (50 ml) at 37 C to a density of 6 x 10^7 cells/ml. The culture was treated as described in materials and methods. Vertical lines denote the times of medium changes: -aa, medium without amino acids; +aa-thy, medium containing amino acids but without thymine; +14Caa, 14C-amino acid mix added (total radioactivity added was 10 uCi/ml at a specific activity of 10 mCi/mmol); +thy, thymine added back to the medium; -14Caa, medium changed to complete medium without 14C-amino acids. Arrows (S1, S2, etc.) show the times at which samples were removed for membrane isolation. □, A540; ■, cell number (determined using the Coulter Counter); ▫, total 14C incorporated; ▽, 14C counts replotted as described in the text.

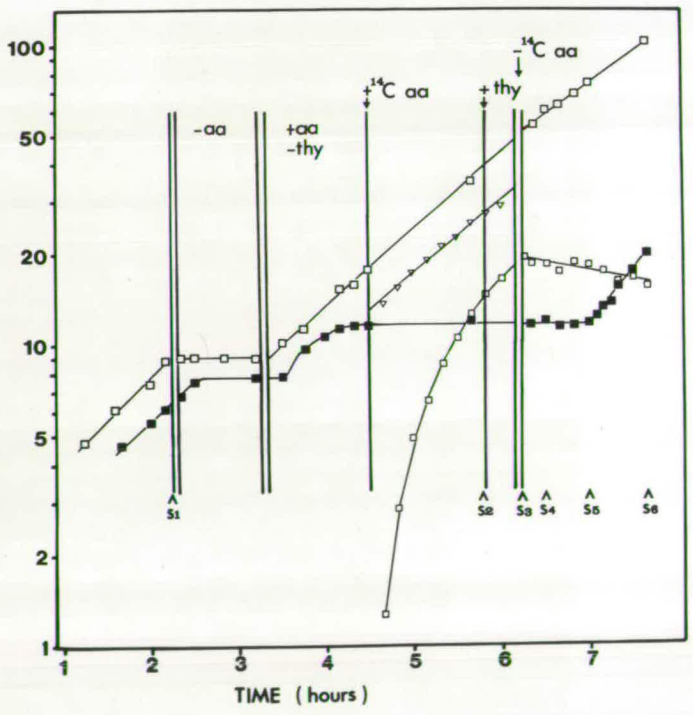


Fig. 13. Staining profile of the crude envelope preparations. Cell envelope samples were solubilized by heating for 4 min at 90 C in solubilization buffer and applied to the gels. Electrophoresis was at 12 mA (stacking) and 20 mA (running). Total electrophoresis time was about 5 hours. After being fixed and stained the gel was scanned at 585 nm using a Vitatron TLD100 densitometer. Sample numbers refer to the times indicated in Fig. 12.

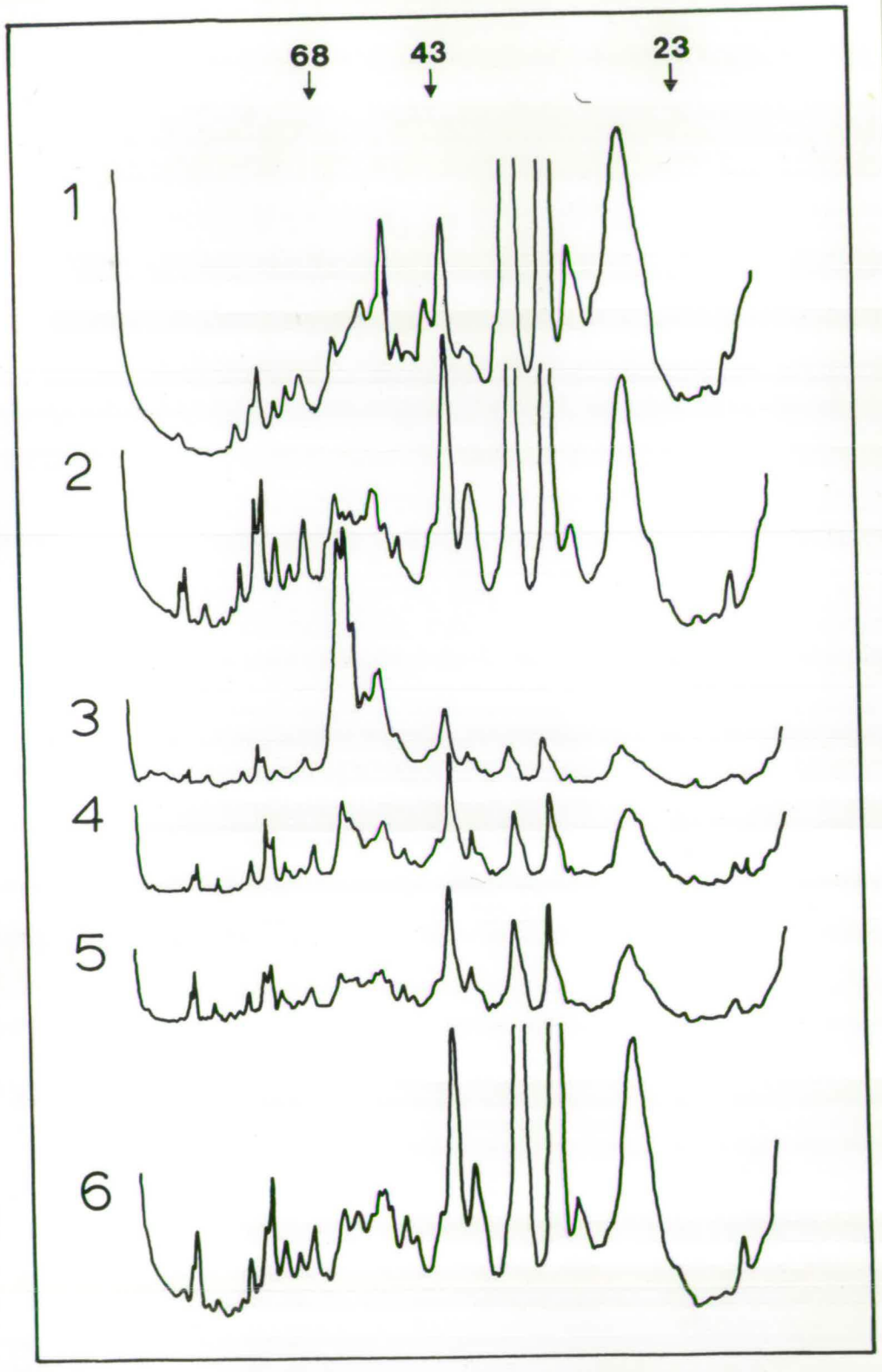

68
43
23
1
2
3
4
5
6

Fig. 14. An autoradiogram was prepared from the stained gel shown in Fig. 13. The autoradiogram was scanned using a Vitatron U6 bandpass filter (400-480 nm).

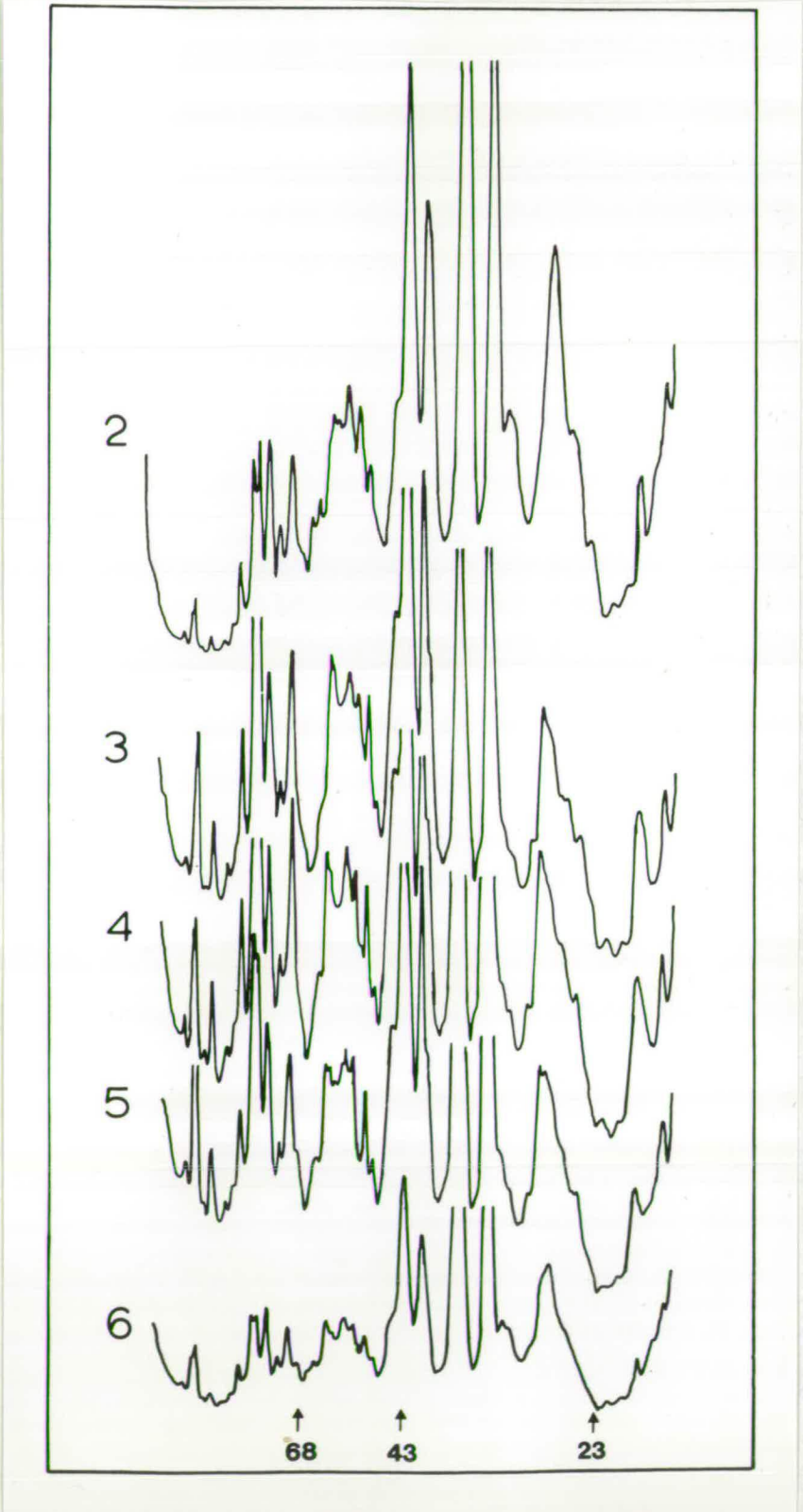


Table V. Specific activity of the membrane samples.

sample number	sp. act. predicted*	sp. act. found**
3	1.00	1.00
4	0.85	0.89
5	0.63	0.68
6	0.43	0.38

* The values given were calculated from the total TCA precipitable counts/min/ml of culture present at the time the sample was taken divided by the total mass (absorbance) of the culture at that time. Each value is expressed as a ratio with sample 3.

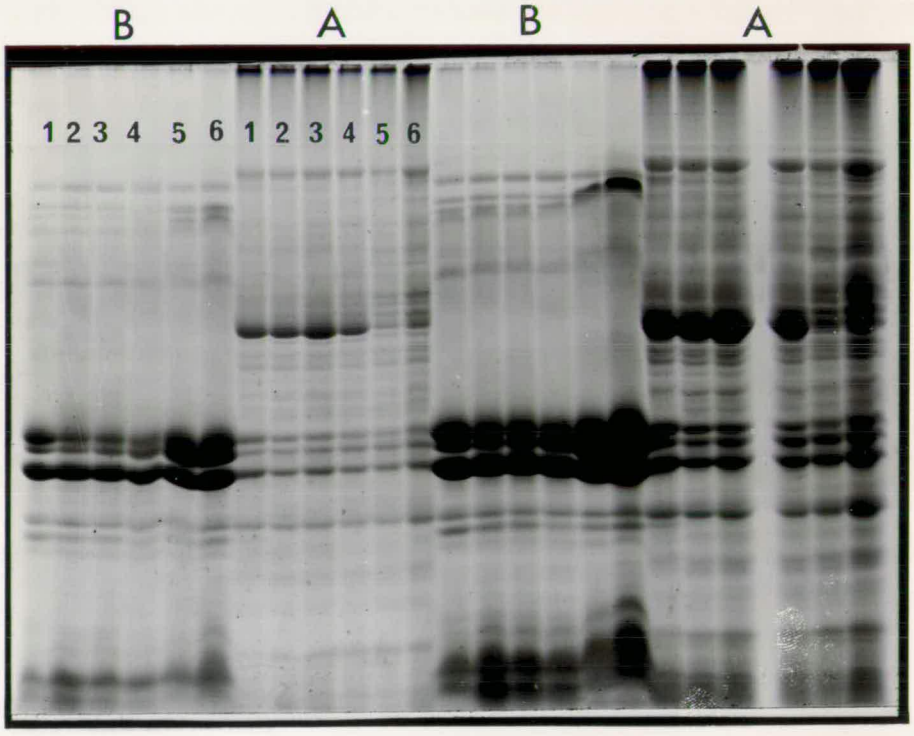
** The values were calculated by integrating the total peak area from the scans of the stained and autoradiographic profiles of the gels to get values for total protein and total 14-C counts in the samples. The values of the protein/14C ratios are expressed as ratios with the protein/14C value of sample 3.

the labelled amino acids were removed.

Since the labelled proteins in this first experiment showed no changes with time it was thought desirable to investigate the dramatic changes seen in the stained profiles. Since the culture was not to be labelled, sufficient material could be prepared to allow the purification of inner and outer membrane fractions. Fig. 15 shows a photograph of a stained gel containing samples of both fractions. It is apparent that none of the changes shown in Fig. 13 occur in these preparations either. On the basis of a number of experiments of this kind, I am forced to conclude that the profiles shown in Fig. 13 are due to some artifact in the procedure for preparation of these membranes; possibly the initiation of DNA replication involves the association of newly formed proteins with the membrane which are removed by the further treatments invoved in purifying the inner and outer membranes.

Some changes are apparent in the fractions shown in Fig. 15. However, they again do not seem to fit the pattern that would be predicted on the basis of an initial protein composition (sample 1) being altered by the treatment to a new form (sample 2) before returning to the initial pattern as the culture completes the imposed synchronous round of DNA replication and cell division. The synchronous D period of the culture should begin at about the time that sample 4 is taken, and by sample 5 the population has begun physical separation of the daughter cells and should be resuming normal asynchronous exponential growth. Yet it is only through samples

Fig 15. Staining profile of purified inner and outer membrane fractions. A culture (2 l in volume) was treated as described in the legend to Fig. 13, except that no 14C-labelled amino acids were added. Inner and outer membranes were purified from the samples by the method of Osborn et al (1972) and the fractions were electrophoresed and stained. A 1-6, inner membrane, S1 - S6; B 1-6, outer membrane, S1 - S6. Each sample is shown twice, at two different levels of protein applied to the gel.



4, 5, and 6 that the membrane profiles begin to change from the norm of sample 1. The pattern shown was very consistent. The significance of this result is not clear at this time, but it is clear that the impressed synchrony has resulted in some disturbance of the protein composition of the membrane, probably through some long-term imbalance in the relative rates of synthesis of certain proteins. The results to date suggest that this procedure is not a good one to use when studying the synthesis of membrane components through the cell cycle.

iv) Discussion

It is clear from Figs. 13 and 15 that unexpected alterations are occuring in the membrane preparations. Fig. 14 shows that these changes do not affect the proteins which have been incorporated into the membrane during the labelling period, and also that no unique proteins synthesized before the D period are incorporated into the cell wall during the D period. We know that, with one exception, the protein composition of the septum is the same as the lateral wall, so this result is perhaps not surprising. However, there was no apparent increase in the level of the protein previously shown to be specifically associated with the septum. Also, the specific activity of the crude wall preparation decreases at the same rate as the specific activity of the culture as a whole. These results suggest that there is no pool of membrane proteins within the cell.

These results, then, imply that cells dividing in the absence of protein synthesis do so by the rearrangement of existing membrane proteins, either achieving net increase in membrane area by the insertion of lipids alone, or with no net membrane synthesis at all. Cell division has been shown to stop in the absence of phospholipid synthesis (Hechemy and Goldfine, 1971), but the possibility cannot be excluded that lipid synthesis is coupled in some less direct way with cell division. Lipid auxotrophs are drastically affected in many macromolecular synthetic processes and generally lyse rapidly (Henning, Dennert, Rehn, and Deppe, 1969; Hsu and Fox, 1970; Cronan, Roy, and Vagelos, 1970; Hechemy and Goldfine, 1971). In at least one

case cell division appears to continue after fatty acid synthesis has ceased (Henning et al, 1969). On the basis of the evidence available it is not possible to say that net wall synthesis is required for septation to occur.

E. A Hypothesis for the Control of the Site of Septum Formation.

The work with the minicell producing strain P678-54 has been very useful in establishing two points about the control of localization of cell division. First, the processes which determine where a division can occur and the signal for septum formation to begin are independent, in that the potential division site exists prior to the initiation of septum formation, at a fixed point on the cell envelope, and the signal to begin septum formation is not specifically associated with the PDS that has arisen in that cell cycle. In the normal cell cycle of E. coli this is not apparent because only one site is available for the signal to act upon. Secondly, the distribution of subunits is not uniform over the surface of the cell envelope. Localized variation exists, although whether this represents a potential division site or a structural component of the septum is not clear. The results discussed in section I. D. make the latter possibility unlikely.

A great deal of work has been devoted to determining the mode of cell wall growth in E. coli. Many of the results have seemed contradictory. It is now apparent that the gram-negative cell wall is both highly ordered and highly dynamic. Much of apparent conflict can be explained by the apparent high rate of 'internal' turnover; that is, the fact that many, if not most, of the components of the cell wall, even those covalently bound to their neighbours, are removed and reinserted at a different location at a very high rate. Each component

seems to have a different rate of turnover (see below) and there are differences in the mode of insertion of 'structural' and other components of the cell envelope.

The cell wall of gram-negative organisms such as E. coli is a complex structure with 3 distinct layers: the inner (cytoplasmic) membrane, the sacculus, and the outer membrane. The inner membrane has a composition similar to that of most other biological membranes, containing about 30% (dry weight) phospholipids, 62% protein, and small amounts of lipopolysaccharide (Osborn et al, 1972; Martin and MacLeod, 1971). The outer membrane is less typical, containing only 13-18% phospholipid, while about 25-40% of the membrane consists of lipopolysaccharide (Osborn et al, 1972; Forsberg, Costerton, and MacLeod, 1970; Schnaitman, 1970; White, Lennarz, and Schnaitman, 1972). Much of the work on cell envelope composition and function has recently been comprehensively (though not always accurately) reviewed by Costerton, Ingram, and Cheng (1974). The sacculus, sandwiched beween the inner and outer membranes, is a polymer consisting of repeating disaccharide subunits (B-1,4-N-acetyl muramyl-N-acetyl glucosamine) linked by B-1,4-glycosidic bonds. The polysaccharide chains are crosslinked via peptide chains attached to the muramic acid group (through C3 of N-acetyl muramic acid).

Braun, Gnirke, Henning, and Rehn (1973) have recently reviewed the literature on the structure of the sacculus and have suggested a 3-dimensional structure based on their own analysis of the amount of murein present in relation to the surface area of the cell. Their

model implies an asymmetrical structure, with the peptide crosslinks to one side of the plane established by the polysaccharide. A similar asymmetry with respect to the 'inside' and 'outside' of the outer membrane has been suggested by Bragg and Hou (1972). They have shown that certain proteins are sensitive to trypsin action in intact outer membranes. This specificity is maintained in outer membrane reconstituted from Triton-X100 solubilized outer membrane, but is lost when the outer membrane is made permeable to large protein molecules.

The characterization of the cell envelope of E. coli is hardly begun, but in view of the complexities already apparent it is not surprising that no simple pattern emerged from early studies on the mode of cell wall growth.

Beachey and Cole (1966) and his colleagues studied the distribution of surface antigens during growth of several species of bacteria including E. coli. Cells were grown in the presence of fluorescent antibodies directed against the cell surface and then transferred to unlabelled homologous antibody (in other experiments this sequence was reversed). Over successive generations the labelled antibodies attached to the cell surface were seen to be distributed evenly among progeny cells without any indication of localized growth of the wall. Only the label attached to the cell pole was conserved in its original location and concentration during successive generations. In one experiment where cells were grown in the presence of chloramphenicol, zones of labelled and unlabelled wall were seen alternating along the length of the cells but even in this case there

were a very large number of such zones per cell.

Van Tubergen and Setlow (1961) specifically labelled the mucopeptide layer by growing the cells in the presence of 3H-diaminopimelic acid (DAP) and then used autoradiography to follow the distribution of this label among the progeny cells. Similar experiments were performed by Lin, Hirota, and Jacob (1971) using growth of the cells in methylcellulose (Lederberg, 1956) to retain progeny cells in chains. Both groups of workers came to the conclusion that growth of the mucopeptide layer must be taking place by intercalation at a very large number of sites.

Recently, Ryter, Hirota, and Schwarz (1973; also Schwarz, Ryter, Rambach, and Hirota, 1974) using improved techniques for preparation of the mucopeptide for autoradiography and very short labelling periods (ca. 1/8 of a generation) have demonstrated that the label is in fact initially incorporated in a narrow band in the center of the cell. A brief chase, however, results in a random pattern of label in the mucopeptide. Thus even though there is apparently a single site within the cell where new material is covalently incorporated into the mucopeptide, the bonds are highly labile and once incorporated at the central site the DAP label is readily transfered to other areas of the sacculus. Hartmann, Bock-Henning, and Schwarz (1974) and Hakenbeck, Goodell, and Schwarz (1974) have shown that the enzymes presumed to be responsible for extension of the mucopeptide are associated with the cell membranes and are prevented from reacting freely with their substrate under normal conditions. This suggests that sites of action

for the enzymes could be restricted to a part of the cell surface, although the enzymes themselves seem to be distributed evenly over the whole cell surface (Goodell, personal communication). One of the enzymes, which acts in vitro as an endopeptidase, is restricted to the inner membrane. This may reflect an asymmetry in murein strucure such as that hypothesized by Braun et al. (1973), with the polypeptide chains oriented toward the inside of the cell.

Lin etal (1971) also followed the distribution of label located in the lipids of the envelope (using 3H-glycerol or 3H- oleate to label the cells) and found no evidence of conserved areas of lipid of any significant size. A similar result was obtained by Green and Schaecter (1972) who also showed that the labelled lipids were partitioned into minicells over many generations in proportion to the amount of label remaining in the cells. There was some suggestion from their data that small blocks of phospholipid were conserved, and they were able to estimate the size of these blocks as about 40,000 nm (about 250 segregating units / cell). Diffusion rates for membrane phospholipids in bacterial and eukaryotic membranes have been estimated by a number of workers and a figure of about 3×10^{-8}-5×10^{-9} cm^2 /sec has been obtained (Lee, Birdsell, and Metcalfe, 1973; Sackmann, Trauble, Galla and Overath, 1973). If blocks of phospholipid are preserved then in this light they must represent only a part of the total membrane phospholipid. On the other hand, a figure of 3×10^{-13} cm^2 /sec has been given for the diffusion constant of lipopolysaccharide in the outer membrane (Muhlradt, Menzel, Golecki, and Speth, 1974). It seems to be apparent that labelling experiments

will have to be very specific before the results are meaningful.

Tsukagashi, Fielding, and Fox (1971) and Wilson and Fox (1971) have performed similar experiments on the segregation of generally labelled lipids among progeny cells (with similar results) and have extended the work to include the distribution of certain membrane-associated proteins (Wilson and Fox, 1971). They again found no evidence for any localization of cell wall growth. As will be seen below, more recent results provide a ready explanation for these results in terms of the mode of insertion in the cell envelope structure of new materials: in general it seems that non-structural proteins at least are inserted at random in the cell membrane (although there is one apparent exception to this rule); if the distribution of already incorporated material is followed a very different picture emerges in many cases.

Thus Autissier, Jaffe, and Kepes (1971), Autissier and Kepes (1972) and Autissier (PhD. thesis, 1971) have presented an elegant series of experiments in which they show that various permeases segregate in a few large blocks among a cell's progeny. They have also shown that the incorporation of the molecules, with the possible exception of the maltose transport system, takes place at random over the whole cell membrane. The basis of their approach was to find a treatment which would differentially kill cells carrying a particular permease and cells without the permease. For example, cells without galactose permease do not grow initially in the presence of galactose as sole carbon source and are consequently resistant to killing by

penicillin. Cells with permease are killed much more rapidly under these conditions. The kinetics of lysis of a mixed population of cells (some preinduced for the transport of the carbon source present and some not preinduced) in the presence of penicillin will be biphasic and the relative proportions of cells with and without permease can therefore be estimated from the shape of the curve. Cells were preinduced and then transferred to a non-inducing growth medium. At times thereafter corresponding to 0,1,2,3, or more mass doubling times, aliquots of the culture were exposed to penicillin and the kinetics of lysis determined. It was found that all the cells behaved as if they retained permease until, after a number of generations which depended on the growth rate of the original culture, 50% of the cells became resistant to killing. Further tests showed that this 50% of the culture contained few, if any, of the permease molecules of the original population. Quantitatively identical results were obtained with permeases for lactose, melibiose, glucose-6-phosphate, maltose, dicarboxylic acids, and glucuronate, as well as phosphotransferase, nitrate reductase, and some less-well characterized systems. In minimal medium, all of these membrane markers were found to be lost from 50% of the cells at the third doubling in mass after transfer to the non- inducing medium. In rich medium, all the cells retained permease at the third doubling, but 50% of the cells had no permease at the fourth doubling. These observations are interpreted in terms of a model in which cells growing in minimal medium (salts + glycerol) have a single central zone of membrane growth and cells in rich medium (nutrient broth) have two zones of growth at equal intervals along the cell. (This interpretation was preferred but they noted that other

models can be fitted to their data.)

Leal and Marcovich (1971) and Begg and Donachie (1973, and personal communication) have studied the distribution of phage T6 receptor sites among progeny cells. These sites are associated with the outer membrane of the E. coli cell wall. Leal and Marcovich followed the kinetics of appearance of T6-resistance among recombinants after mating a tsx- hfr donor with a tsx+ recipient. The tsx locus is closely linked with the proC locus and the fraction of phenotypically T6-resistant cells amongst recombinants receiving the proC+ gene was therefore scored at intervals after mating. (Donors were selected against with streptomycin.) As had originally been reported by Hayes (1957) resistance was not expressed immediately after the entry of the tsx- allele but only after a period of cell division. Leal and Marcovich used the fact that penicillin will preferentially kill dividing cells at appropriate concentrations (Lederberg, 1956) to show that the appearance of T6-resistance required cell division. These results were difficult to interpret quantitatively because of the uncertainty of the time at which the recipient cells become genotypically tsx-. However, the main rise in T6 resistant cells, from 0 to close to 50%, took place during the 2 1/2th to 3 1/2th doubling in numbers of the recombinants. Since the parent cells were grown in minimal medium this result is very similar to that of Autissier et al (1971) and could be interpreted to indicate a similar mode of growth for the outer and inner membranes.

Begg and Donachie (1973) utilized an amber tsx- mutant together with a temperature sensitive suppressor mutation (Smith, Barnett, Brenner, and Russell, 1970) to study the same problem. Segregation of T6 receptors was observed by exposing the cells to T6 phage and observing the distribution of phage on the cell surface using the electron microscope. Their results were not inconsistent with those of Leal and Marcovich, but showed that cells growing in minimal medium possessed a single polar growth site rather than the central site postulated for the inner membrane by Autissier et al (1971). Both Leal and Marcovich (1971) and Begg and Donachie (1973) noted that the reverse experiment in which cells originally of the tsx- phenotype were made phenotypically T6-sensitive showed that new phage receptor sites could be inserted at random over the whole cell surface. This is similar to the finding of Autissier et al regarding the insertion of new permease molecules into the inner membrane.

Autissier (1971) also followed the distribution of parental phospholipids relative to the distribution of parental transport systems in populations growing in the absence of inducer. The result she obtained depended on the way in which the phospholipids were labelled. If the lipids were labelled with 3H-glycerol or with 32P then at the end of the third generation most (75-85%) of the label was contained in the cells which still possessed the permease (ie., 50% of all the cells). However, if the labelling had been carried out using 14C-acetate or 14C-oleic acid then the label was found to be equally distributed among permease- containing and permeaseless cells at the end of the third generation. This was interpreted to mean that the

fatty acid side chains of the phospholipids were undergoing rapid translocation within the membrane, while the glycerol moiety was relatively more stable. These results seem to contradict those of others (see above) but they do suggest that it is not a simple procedure to determine the mode of growth of the cell membranes using radioactive label. It has been shown by others (Ballesta and Schaechter, 1971; Ballesta, deGarcia, and Schaecter,1973) that the different phospholipids of the E. coli membrane are subject to differential control in their rate of synthesis and that the non-acylated glycerol of phosphatidylglycerol is much less stable (subject to a greater degree of turnover) than the acylated glycerol.

There are a number of reports which indicate that singularities exist in the cell envelope of E. coli. Schwarz, Asmus, and Frank (1969) showed that high concentrations of penicillin caused the splitting of the mucopeptide layer of E. Coli at a localised site. The location of this site as a function of cell length has been mapped by Donachie and Begg (1970) using phase-contrast microscopy of cells immobilized in thin layers of agar containing high concentrations of penicillin. The splitting of the mucopeptide layer is followed by the appearance of the contents of the cell, bounded by the membrane, as a spherical body. In the presence of Mg++ ions this spheroplast is stable and its point of origin can be located with some accuracy. In these experiments they found that the 'penicillin-sensitive site' in the sacculus is not randomly located but its location bears a very precise relation to cell size.

In cells growing in any particular medium increase in cell volume during the cell cycle appears to take place entirely by increase in length, without any change in diameter. In addition, cell length can be increased, again without any increase in cell diameter, by specifically inhibiting cell division for a period of of time. In a series of such experiments (some published in Donachie and Begg, 1970, but most unpublished) cell division was first inhibited (usually by growth in low concentrations of penicillin, eg. 15 IU/ml) and then allowed to resume after variable increase in cell length (usually by adding penicillinase to the medium). Cells were then spread on agar layers and the locations of the first and subsequent divisions were measured. Division did not resume synchronously in any given cell but the intervals between succesive divisions at different sites in the filaments were less than the doubling times for cell length so that all cells eventually returned to the normal lengths for the particular medium. The numbers and positions of division sites found in this way were determined. The most striking finding is that the number of sites/cell increases in a sharply discontinuous manner with increasing cell length. A jump in the number of sites/cell occurs at each doubling of a minimum cell length, that of the normal dividing cell in the particular medium used (salts + glycerol). The length of the daughter cells arising from this division is defined as one 'unit length'. The number of division sites increases only at each doubling in number of this basic unit length. The sites were always equidistant from one another along the length of the cell.

When such experiments were repeated, with the difference that instead of adding penicillinase to allow division, more penicillin was added to induce the splitting of the mucopeptide, then the distribution of the penicillin-sensitive sites was found to be essentially identical to that of the division sites. This supported the earlier conclusions (Lederberg, 1956; Schwarz etal, 1969) that the penicillin-sensitive site in cells is the division site. The differences observed between numbers and positions of division sites and numbers and positions of penicillin-sensitive sites were that the penicillin-sensitive sites observed in filaments generally corresponded only to the positions of the first divisions observed in filaments (and division takes place preferentially at what are probably the oldest division sites: see Donachie and Begg, 1970) and that penicillin- sensitive sites could be observed in cells that were less than 2 units in length (ie., were too small to divide during the normal course of events). These sites were found in cells of all lengths and at a position dependent on cell length. In cells of 1 unit in length (ca. 1.7um long in the strain used) this site was at or very close to one pole. As the length increased to 2 units, the site was found to remain at a fixed distance from one pole (one unit length away) until in cells of 2 units it was in the centre of the cell.

Such observations suggested that the division site arose during cell growth close to the position of a preexisting site and that growth of some part of the cell envelope took place asymmetrically at that new 'potential division site'. Presumably what is revealed by the penicillin technique is the location of a set of autolytic enzymes

which split the murein adjacent to them when net synthesis of murein is inhibited. It is known that murein hydrolases are found in both the inner and outer membranes of the cell wall (Hakenbeck et al., 1974). In this case, an active hydrolase seems to be located at a point corresponding to the interface beween old and new outer membrane revealed by the studies on the segregation of T6 receptor sites.

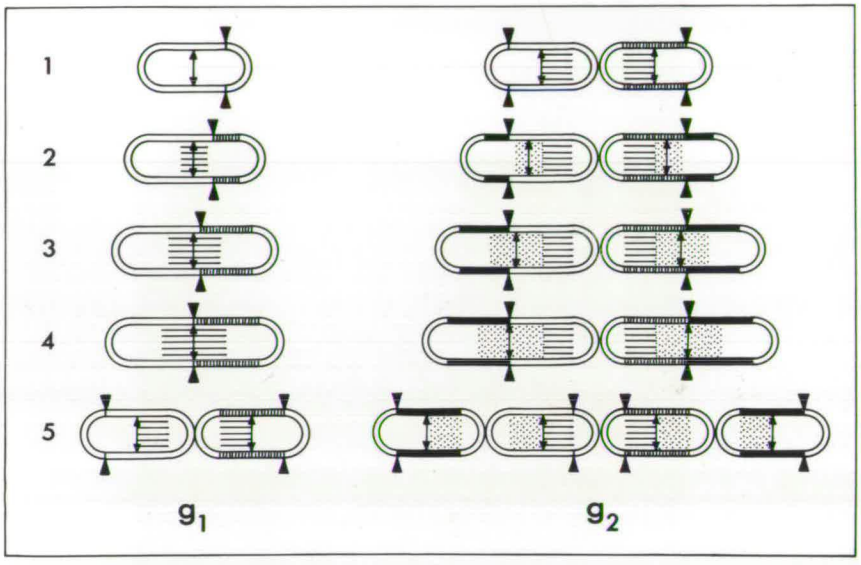
Two structurally unique areas of the cell wall have been described in biochemical terms. Ballesta and Schaechter (1972) have shown that the portion of the inner membrane associated with the chromosome has an altered phospholipid composition. My own work with P678-54, in collaboration with Goodell and Schwarz, has shown that the cytoplasmic membrane of the cell pole (the septum) has a unique protein composition and also differs in phospholipid composition from the rest of the cytoplasmic membrane. Whether these discontinuities are involved in the establishment of localized growing zones remains a matter of conjecture.

Two distinct mutants of E. coli have been described which might be specifically affected in the localization of the division site. One of these, CRT12 (Hirota, Jacob, Ryter, Buttin, and Nakai, 1968) has not been well characterized, but the mutation also affects DNA segregation. If DNA segregation is effected by localized growth of the cell wall, as suggested by Jacob, Brenner, and Cuzzin (1963), then this would be expected if the site of septum formation was determined by localized growth of the cell wall and this mutant was abnormal in the pattern of cell wall growth. The other mutant, P678-54, has

already been discussed. In this case, the pattern of cell wall growth would not be expected to be abnormal. Analysis of this mutant's division pattern suggested that the localization of the potential division site is independent of the process of division; that is, the potential division site is a defined point on the cell envelope where septation can be initiated by an independently controlled sequence of events, and it is the initiation of division which is abnormal (section B; also Teather, Collins, and Donachie, 1974).

A reasonable model for the growth of the outer membrane can be devised on the basis of the data for the segregation of T6 receptor sites and this model is compatible with the results of Autissier et al (1971) and Donachie and Begg (1970). This model is shown in Fig. 16 (outer layer). The data of Hirota etal (1973) for the growth of the sacculus requires an alternative model (Fig. 16, inner area), which is also compatible with the data of Autissier et al. for the inner membrane. It is difficult to see how the data of Hirota et al. and Donachie and Begg could be reconciled. It should be emphasized at this point that some or all of the constituent molecules of the envelope could undergo rapid turnover without affecting the dimensions or spatial location of the multimolecular structure as a whole. An appropriate analogy here is the genome of the cell. In the bacterial chromosome the sequence of bases is permanent but repair synthesis can effect the substitution of any one base for any other identical base, even though net synthesis of DNA takes place at only two 'growth sites'. If repair synthesis were much more active than it in fact is, then the conservative replication of DNA could not easily have been

Fig. 16. Model for the control of Localization of Cell Division in E. coli. Two generations of growth are shown for a cell with a generation time >60 minutes. The outer area of the cells represents the outer membrane; the inner area represents the sacculus. The shaded areas represent newly synthesized material.



demonstrated by the classical labelling experiments that were used to do so. This would be especially true if excised bases were reutilised, both for further repair and for replication, in preference to exogenously supplied bases.

Since there is no known permanent bond between the layers of the cell envelope (the one well characterized link, between the sacculus and the outer membrane (Braun and Bosch, 1972, 1973; Bosch and Braun, 1973) has been shown to be subject to rapid turnover (Inouye, Shaw, and Shen, 1972)), it is not unlikely that the different layers of the cell wall do in fact have the different modes of growth shown in Fig. 16. If this were the case then it would in fact provide a very precise means of regulating the position of the septum. The weakness of the individual models shown in Fig. 16 is that they require an independent mechanism to measure the amount of growth that has occurred. If the two modes of growth shown are combined in a single cell then a mechanism is created wherebye the cell can 'know' that it has precisely doubled in size. In the diagram the inner area of the cells represents the sacculus, the outer one the outer membrane. The shaded areas show the regions within which net synthesis of wall material occurs, while the arrows show the location with time of the 'active' sites as they they are moved relative to oneanother by the independent growth of the two layers. It will be seen that after one doubling the sites coincide: if, for example, each site had associated with it part of the enzyme system necessary for septum formation then the point at which they come together becomes the point at which the complete enzyme system is created, and hence the point at which septum

formation is initiated. Net synthesis for the next cell cycle is initiated at new sites defined by the interface between 'old' and 'new' wall material, just as in the case of S. faecalis (Higgins and Shockman, 1971; Higgins, Daneo-Moore, Boothby, and Shockman, 1974).

The predictions of this model are reasonably easy to test. Firstly, it must be confirmed that the outer membrane has a different mode of growth from that of the inner membrane and cell wall. This should be readily checked by combining experiments of the type done by Autissier et al with experiments on the segregation of phage T6 receptor sites. In this case, one would look to see whether the population that is resistant to penicillin treatment has T6 receptors. Another possibility is to examine the mode of cell wall growth of mutants with abnormally located divisions, such as CRT12 (Hirota et al, 1968), which should show an altered pattern of cell wall growth. Work currently in progress in the laboratory of Dr. U. Schwarz should also help to confirm or reject the model. The work involves the isolation of the enzymes involved in murein synthesis, and they intend to prepare ferritin-conjugated antibodies to these proteins and attempt in that way to visualize the location of the enzymes within the cell wall.

II. The Timing of Cell Division.

A. Introduction.

In *E. coli* any treatment which interferes with DNA replication (eg., ultraviolet (UV) irradiation, thymine deprivation, treatment with drugs such as nalidixic acid or mitomycin C) blocks cell division without initially affecting cell growth, so that abnormally long filamentous cells are formed. It is clear that cell division is in some way coupled to chromosome replication. As mentioned above, the block to cell division is not immediate in an exponentially growing population: cells which have completed a round of chromosome replication within the preceding 20 minutes divide. This implies that termination of a round of chromosome replication either relieves a block on the PDS or provides a positive signal for division to commence.

The idea that termination of a round of chromosome replication provided a positive signal for cell division was first advanced by Clark (1968). The model provided an explanation for the timing of most of the known events of the cell cycle in *E. coli*, and could also be used to explain the fact that cell size varied with growth rate. The discovery that there was a constant period of protein synthesis which was also required to allow the cell to initiate septum formation (Pierucci and Helmstetter, 1969) removed much of the force from this argument for a positive signal at the termination of chromosome

replication.

Other findings subsequently made such a signal seem unlikely. First, there are a number of mutants known which continue to divide and produce normal sized, anucleate, progeny cells in the absence of DNA replication (Hirota, Jacob, Ryter, Buttin, and Nakai, 1968; Hirota, Ryter, and Jacob, 1968; Inouye, 1971). This indicates that the cell has some means independent of chromosome replication to control the localization, and hence the timing, of cell division. Jones and Donachie (1973) have shown that the cell can in fact complete almost the entire cell cycle, presumably including most of the events of the D period, before termination of DNA replication takes place: under appropriate conditions the D period can be reduced to about 5 minutes. (They have also shown that there are 2 proteins associated with the cytoplasmic membrane that appear to be synthesized specifically at the time of termination, so there could well be a positive signal made to indicate that chromosome replication has been completed: N. C. Jones, personal communication.) Thus while termination is necessary for division to take place, the timing of this event seems to play no role in either the timing or localization of cell division. The problem of defining the nature of the link beween chromosome replication and cell division remains.

B. The interaction between bacteriophage P1 and cell division.

i). Introduction.

If the replication of certain episomes is blocked, either by irradiation prior to their introduction to the cell (Monk, 1969) or by using mutants that are temperature-sensitive for DNA replication (Scott, 1970 (a P1 mutant); Otsuji, personal communication (a mutant F factor)), cell division is blocked. I have investigated the kinetics of filament formation in E. coli, using UV damaged bacteriophage P1 to block cell division . While the results to date are only preliminary, they suggest that blocking the replication of the P1 genome interacts with the cell to block division in the same way that blocking chromosome replication does.

ii). Materials and Methods.

Bacteria, phage, and growth conditions. All experiments were carried out using E. coli K12 strain W3110 ($su^-thy^-lacY14amb^-str^r ilv^-$) and a uvrA6 derivative. Both strains were obtained from Dr. M. Monk. $RecA^-$ derivatives were prepared from both of these strains by mating with an Hfr donor that transfers the recA marker within 10 min of mating (E. coli K12 strain JC5088; the relevant markers are $recA56^- thy^- str^S$ KL16Hfr: obtained from Dr. P. Broda). Thy+ recombinants were selected and tested for the coinheritance of the recA marker by their greatly increased sensitivity to UV and by their sensitivity to methyl methane sulphonate(MMS sensitivity was tested using plates containing 0.0003 % (v/v) MMS in LB agar, as described by Monk and Kinross (1972)). The P1 strains used were P1kc (Lennox, 1955), P1CM (Kondo and Mitsuhashi, 1964), and P1vir (Ikeda and Tomizawa, 1965), all obtained from Dr. M. Monk.

Cultures were grown at 37 C in LB or in 007 minimal medium (Clark and Maaloe, 1967). 007 was supplemented as required with 50 ug/ml isoleucine and valine, 40 ug/ml thymine, and 2 mg/ml glucose. For phage infection, cultures were spun down and resuspended in about 1/10th of the original volume of the growth medium, $CaCl_2$ was added to give a concentration of 5mM Ca^{2+}, and the phage were added. The suspension was incubated at 37 C for 10 minutes and was then diluted back with the remainder of the original growth medium. Phage stocks were prepared by the plate lysis technique (Lennox, 1955) and concentrated when necessary by centrifugation in a Beckman L2-65B

centrifuge, using the SW36 (40 min at 36,000 rpm) or SW19 (240 min at 19,000 rpm) rotors, onto a shelf of 60% Urografin (Schering Chemicals Ltd.). The phage were stored in the Tris-ammonium acetate-vagnesium sulphate buffer described by Walker and Anderson (1970).

Ultraviolet irradiation. Bacterial cells in unsupplemented 007 salts or phage P1 in phage storage buffer were irradiated in glass petri plates (total volume 5 ml) for various times with UV (Hanovia Bactericidal Ultraviolet Unit). The dose rate was determined using a Latarjet dosimeter.

Measurement of cell size. Cell length distributions were obtained by spreading aliquots of the culture on slides coated with agar containing 0.05% NaN_3 as described in section I. B.

Electron microscopy. Cells were fixed and stained with OsO_4 in Kellenberger buffer as described in Kay (1965). The fixed cells were dehydrated in an ascending series of acetone/water (30, 50, 75, 90, 100, and 100% acetone). They were then embedded in Araldite (Glauert and Glauert, 1958) and thin sections were cut. Sections were examined using a Siemens Elmiskop 1A electron microscope.

iii). Results.

Irradiation of phage P1CM with a dose of 3000 ergs/mm^2 was sufficient to inactivate over 99% of the plaque forming units (pfu) as assayed on strain W3110. When W3110 is infected with phage that have been irradiated at this level at a multiplicity of infection (moi) of 10, Over 90% of the population consists of filaments after 2 generations of growth (the stated moi in all cases is based on the number of pfu present in the phage stock prior to UV irradiation, assayed by plaque forming ability on strain W3110). After a further 2 generations of growth the population is much more heterogeneous. The number of filaments per ml of culture is reduced and most of the remaining long cells contain visible septa. This suggested that the block to cell division which was induced by the irradiated P1 might be relieved by repair of the P1 genome, leading either to vegetative growth of the phage and cell lysis, destruction of the phage genome, or lysogeny and the subsequent division of the cells. Accordingly, the effect of UV irradiated P1 on an excision repair (uvrA$^-$) strain was investigated.

In contrast to the above result, when strain W3110uvrA6 is infected with UV irradiated P1 the number of filaments in the culture does not decrease with time (see Fig. 22; 'total filaments'). The block to cell division is permanent in this strain: filaments greater than 50 times the normal average length of strain W3110uvrA6 under these growth conditions are produced, and in no case has a septum been observed in a filament in these populations. The effect is the same

with P1kc, P1CM, and P1vir. RecA derivatives of both strain W3110 and W3110uvrA6 also filament when infected with UVed P1, although in these strains the block is not permanent in either case. Thus the block to cell division is relieved by the recA mutation, just as recA allows cells in which chromosomal DNA replication has been blocked to continue dividing (Inouye, 1971).

Fig. 17 shows the effect on cell growth of infection of W3110uvrA6 with P1 irradiated with various levels of UV. P1 infection blocks cell division in all cases. The effect of the phage on cell growth is marked at lower doses of UV. Cell diameter increases, large vacuoles appear within the cells, and cell lysis occurs (the extent and timing of lysis was extremely variable). At a dose of 2000 ergs/mm^2 or greater very few cells were affected in this way (at this moi).

Since the cells grew into very long filaments when infected with UVed P1 it was of interest to see whether the block could be imposed and maintained by a single P1 genome. Cells were infected with UVed P1 at various moi's and the proportion of filaments in the population was determined after 4 generations of growth. The number of filaments at this time was taken to represent the number of cells at the time of infection in which division was blocked: the number of normal cells after 4 generations of growth was divided by 16 to give the number of uninfected cells at 0 time. Table VI shows the experimental data and the calculated proportion of the population which did not receive a 'hit' (infection with an UV damaged P1 genome leading to filamentation). When these results are plotted (Fig. 18) the result is

Fig. 17. The effect of UV irradiation of P1 on the growth of an infected culture. E. coli strain W3110uvrA6 was grown in LB to a density of 3 X 10^7 cells / ml. Aliquots of the culture were then infected with P1CM irradiated with a total UV dose of 0 (+), 400 (●), 800 (□), 1200 (■), 1600 (▼), or 2000 (△) ergs / mm at an moi of 5. Open circles represent the uninfected control culture. The vertical line indicates the time of infection. Cell number was determined using a Coulter Counter model A with a 30 um diameter orifice. The optical density (A540) of the culture was measured at 540nm using a Hilger - Watts spectrophotometer.

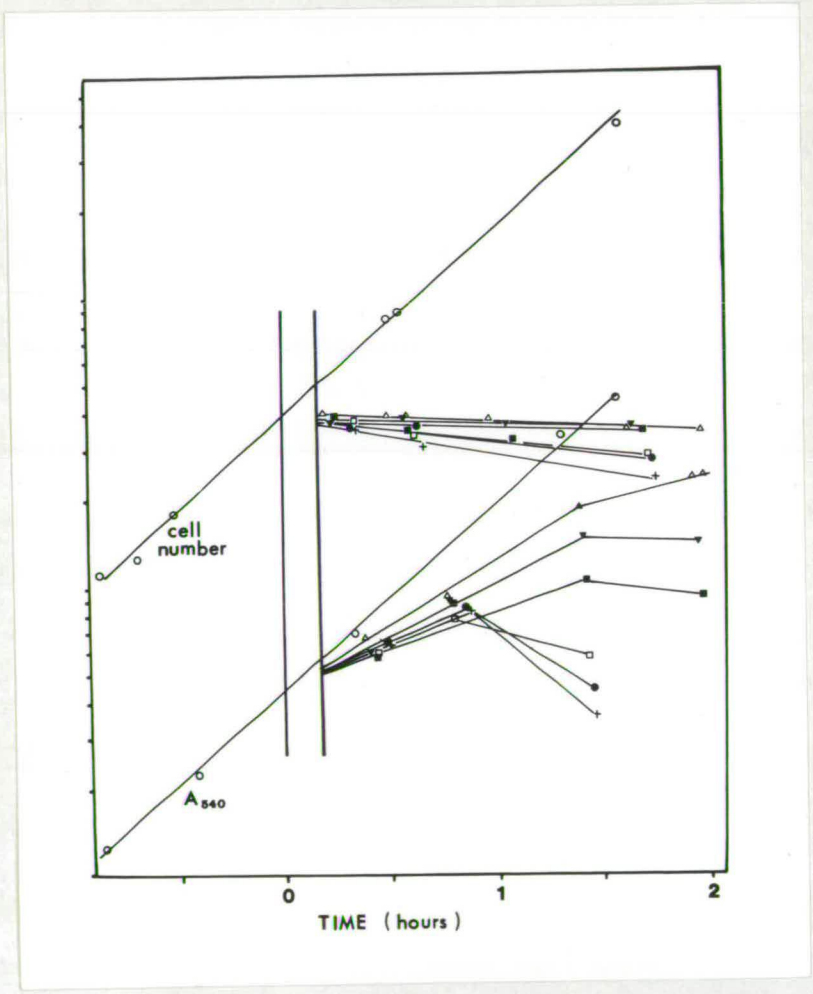


Table VI.

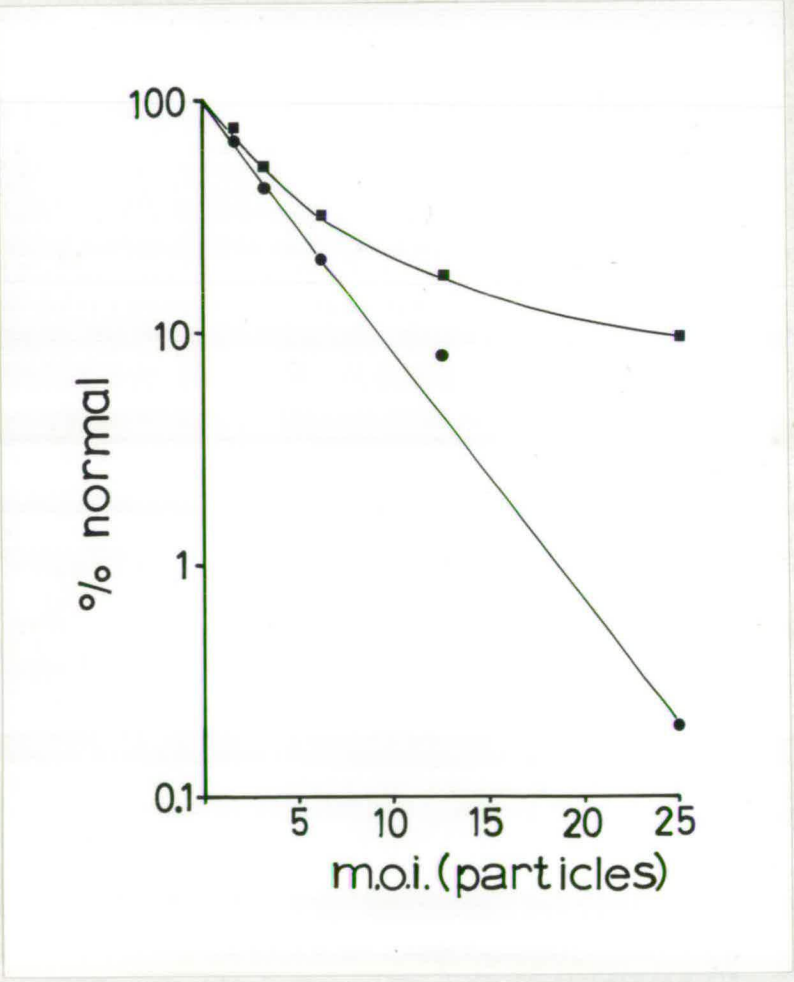
moi	Total cells after 4 generations of growth		calculated number of uninfected cells at 0 time (and as % of total cells at o time)
	normal	filaments	
25	83	48	5 (9.4)
12.5	492	147	31 (17.4)
6.25	310	40	19 (32.2)
3.13	167	14	10 (41.7)
1.56	186	4	12 (75.0)

not consistent with any simple expectation: the probability of an input phage particle causing a cell to filament appears to decrease with increasing moi.

One possible and simple explanation for such a result would be that a part of the bacterial population is resistant to the phage. As shown in Fig. 18 this possibility can be used to manipulate the results to produce single-hit kinetics of the type that would be expected if 1 phage genome was sufficient to block cell division (the assumption could also be used to justify transforming the results into a 'multi-hit' type curve: a figure of 8.8% of the population resistant to the phage was chosen simply because it allows a good approximation to to a straight line in this plot!). There does not appear to be any reason to expect that this would be the case, however. I know of no instance in which a bacterial population has been shown to be heterogeneous in its sensitivity to phage infection. The size distributions for filamenting populations described in Figs. 22 and 23 argue that the entire population must contribute to the filamenting population; ie., that the resistant subfraction could not represent those cells in a particular part of the cell cycle.

A more attractive explanation for the kinetics observed would be that 1 (or 2, or some other finite number) hit was sufficient to cause filamentation, but infection of the cell with 2 or more damaged P1 genomes tended to cause cell death by lysis, with a probability increasing with the number of phage genomes in the cell (or the number of phage attached to the cell). In fact, the sum of the number of

Fig. 18. Dose response curve for filament formation with moi. Aliquots of a culture of E. coli strain W3110uvrA6 were infected with UVed P1 (total dose 3000 ergs/mm^2)at various multiplicities of infection. After 4 generations of growth the cultures were killed by the addition of 1/10 volume formalin. The proportion of filaments and normal cells was determined using a Petroff-Hausser counting chamber (Table VI) and the relative numbers of infected and uninfected cells at 0 time calculated as described in the text. (■) experimental data from Table VI; (●) the same data after subtracting 8.8% from each value to correct for a hypothetical resistant subpopulation.



filaments present after 4 generations of growth and the calculated number of uninfected cells present at 0 time is less than the true number of cells present at that time, and this discrepancy increases with moi. At an moi of 25, the sum of the number of filaments and the calculated number of uninfected cells represents only 43% of the true 0 time population (the moi for viable phage in this case is about 0.025).

While the data available are not complete, it can be shown that they are consistent with the hypothesis that a single phage genome is sufficient to block cell division and that more than one such 'hit' tends to cause cell death. Table VII is a standard Poisson table showing the number of cells that would be expected to receive 0,1,2,etc. hits at a particular moi. Fig. 19 shows a number of theoretical curves derived from this table and fitted to the experimental data. First, the true proportion of the population which received no hits was calculated. When these points are plotted and the line thus established used to calculate the effective moi, they suggest that the effective moi is only about 1/5 the expected moi that was based on the number of pfu present before UV irradiation. This shows that most of the phage particles added to the culture are completely ineffective. If the 'effective' moi is used now as a basis for calculation, one can predict the proportion of the population that should receive 1,2, or more than 1 hit. These curves are also drawn in Fig. 19. I have calculated the proportion of the original population that filamented, at various moi's, and these points are also plotted. They correspond very well with the theoretical curve for cells

Table VII. Poisson Distribution.

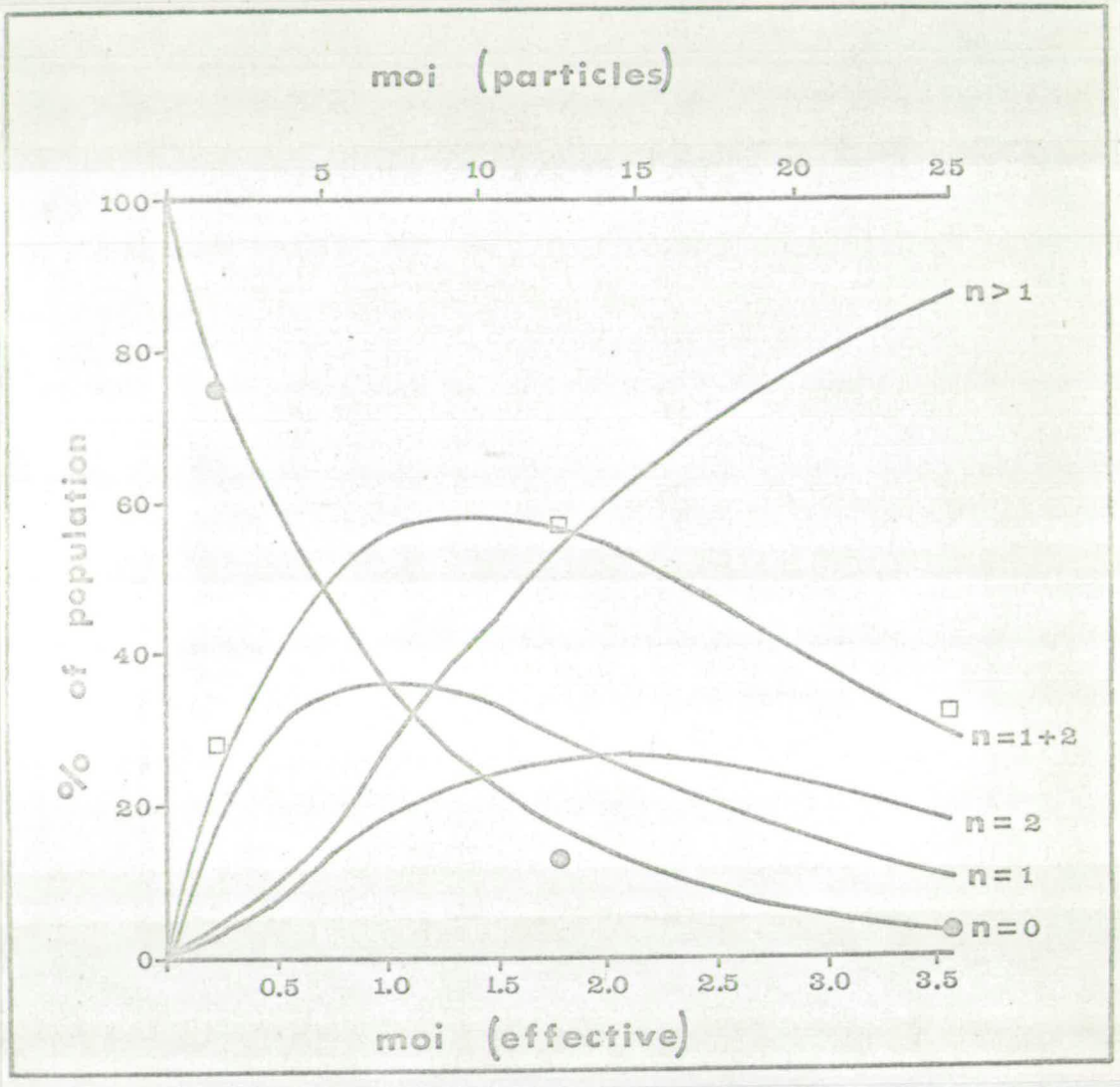
moi($\bar{Y}$)	percent of population receiving n hits*								
	0	1	2	3	4	5	6	7	8
0.5	60.65	30.33	7.58	1.26	0.16	0.02			
1.0	36.79	36.79	18.39	6.13	1.53	0.31	0.05	0.01	
1.5	22.31	33.47	24.10	12.55	4.71	1.41	0.35	0.08	0.01
2.0	13.53	27.07	27.07	18.04	9.02	3.61	1.20	0.34	0.09
2.5	8.21	20.52	25.65	21.38	13.36	6.68	2.78	0.99	0.31
3.0	4.98	14.94	22.40	22.40	16.80	10.08	5.04	2.16	0.81
3.5	3.02	10.57	18.50	21.58	18.88	13.22	7.71	3.85	1.69
4.0	1.83	7.33	14.65	19.54	19.54	15.63	10.42	5.95	2.98

* Calculated from the standard Poisson Distribution:

$$f_{(n)} = \frac{100\bar{Y}^{(n-1)}}{(n-1)!e^{\bar{Y}}} \times \frac{\bar{Y}}{n}$$

where $\bar{Y}$ is the average number of hits/cell, and $f_{(n)}$ represents the percent of the population receiving n hits.

Fig. 19. Theoretical curves for number of cells receiving n hits at various moi. Each curve represents the proportion of the population that should receive n hits at various moi (bottom abcissa). The experimental results have also been plotted, using an altered scale for moi (top abcissa). (●) cells receiving no hits; (□) % of the 0 time population that was permanently blocked in cell division.



receiving 1 or 2 hits, and not at all with the theoretical curve for cells receiving more than one hit. On this basis it would appear that a single phage genome can cause a permanent block to cell division. The correspondence of the experimental data with the curve for 1 or 2 hits is likely to be fortuitous, but it is clear that one 'effective' hit can cause filamentation and that more than one hit will tend, with a probability increasing with the number of hits, to cause cell death (possibly through recombination beween the damaged genomes).

If a single phage genome can block cell division then the block must be independent of any signals initiating or allowing division which may arise from other sources, such as the chromosome of the cell, that division can proceed. If the block caused by the phage is mediated by the same cellular mechanism that couples chromosome replication and cell division, then irradiation of the cell's chromosome with UV should lead to filamentation and ultimately to cell death with a probability that would also depend only on the probability of some UV induced event in the cell's DNA and otherwise be independent of the DNA content of the cell. In other words, it should not be necessary to inactivate all of the cell's chromosomes to kill the cell (in this case, 'death' is measured by plating for viable cells: filamenting cells are dead by this criterion).

Fig. 20 shows a set of theoretical curves for survival vs. UV dose for cells with various numbers of nuclei, based on the following four assumptions: a) all of the nuclei in the cell have to be hit once, or b) all of the nuclei in the cell have to be hit more than

Fig. 20. Predicted dose response curve for UV for cells with varying numbers of nuclei.

a. All nuclei must be hit at least once to kill the cell.

b. All nuclei must be hit more than once

c. Only one of the nuclei must be hit once to kill the cell.

d. Only one of the nuclei must be hit, but it must be hit more than once. The calculations were based on the standard Poisson distribution. The functions used were:

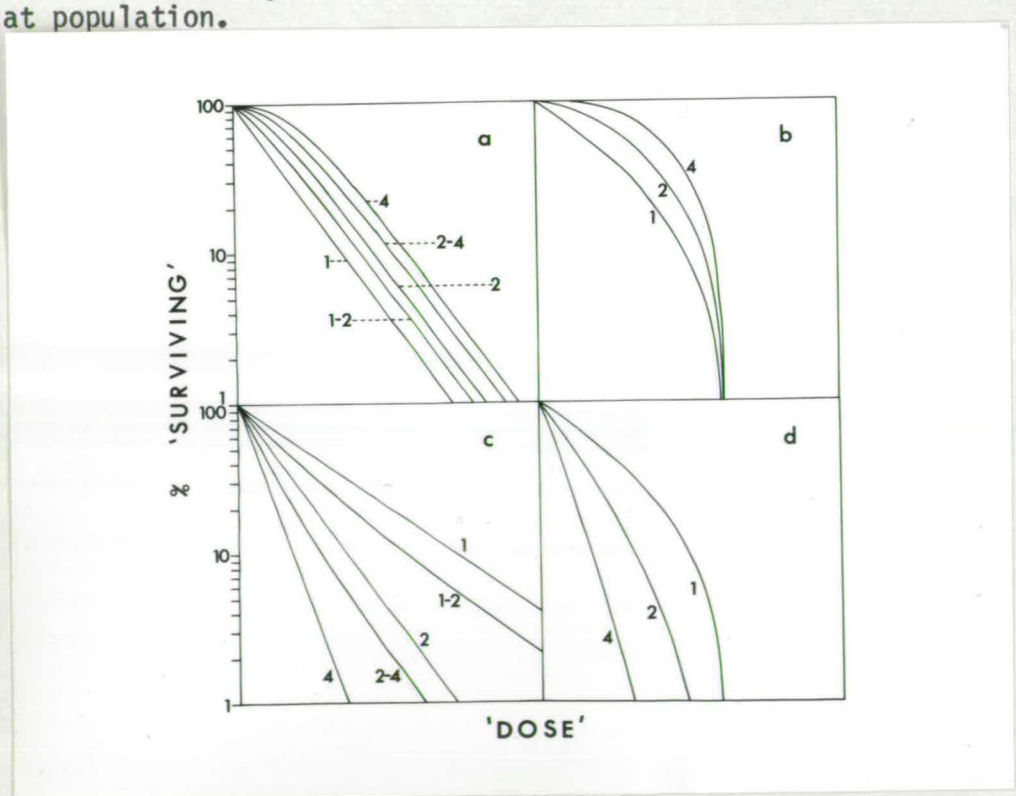
a. $S = 1-(1-e^{-pD})^n$

b. $S = 1-(1-(e^{-pD} + pDe^{-pD}))^n$

c. $S = e^{-npD}$

d. $S = e^{-npD} + pDe^{-npD}$

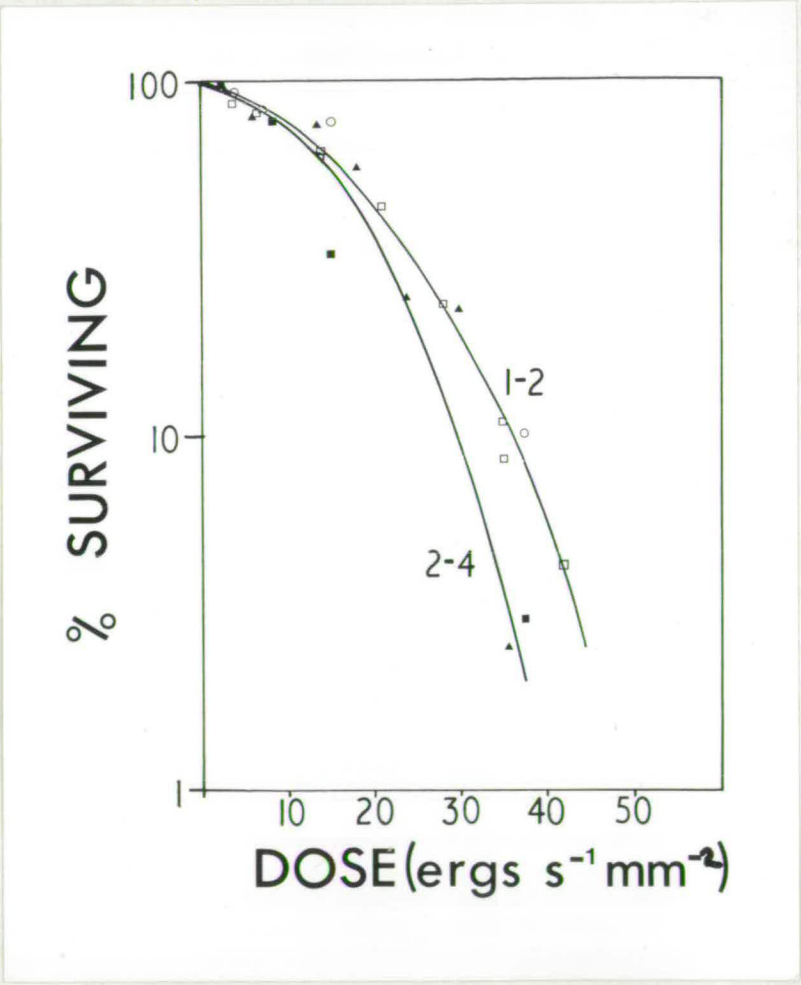
where S is the proportion of cells surviving the treatment, p is the probability that a given dose of UV will damage a given target, D is the dose of UV, and n is the number of nuclei (targets) in the cell. The numbers on the figure refer to the number of targets in the cells in that population.



once, or c) only one of the nuclei in the cell has to be hit once, or d) only one of the nuclei has to be hit, but it has to be hit more than once, in order to kill the cell (c. and d. are indistinguishable from a situation where there is a single 'target' of relative size 1, 2, or 4). Curves a. and b. are distinguished from curves c. and d. by the relative sensitivity of cells with increasing numbers of chromosomes. In the first two cases sensitivity decreases with increasing DNA content, in the second two cases it increases.

Fig. 21 shows the result of an experiment in which low concentrations of penicillin (10 iu/ml, a concentration which immediately inhibited all further cell division without reducing the rate of cell growth) were used to temporarily inhibit cell division (Starka and Moravova, 1967; Starka, 1971), producing populations with varying chromosome complements. If at the time of addition of penicillin the cells contain n chromosomes/cell on average (in the case of 007 grown cells n=1.8, assuming C=40 min and the generation time is 60 min; Cooper and Helmstetter, 1969) then after 1 generation of growth in penicillin the cells contain on average 2n chromosomes. For these experiments both populations were exposed to penicillin for the same length of time. Fig. 21 shows that the population with the greater chromosome content is in fact more sensitive to UV irradiation. At a dose of 40 ergs/mm^2 the population that was grown in the presence of penicillin for one generation has a survival level of only about 1/5 that of the population that was irradiated immediately upon the addition of penicillin. This result, and the general shape of the survival curves, suggests that model d from Fig. 20 is most nearly

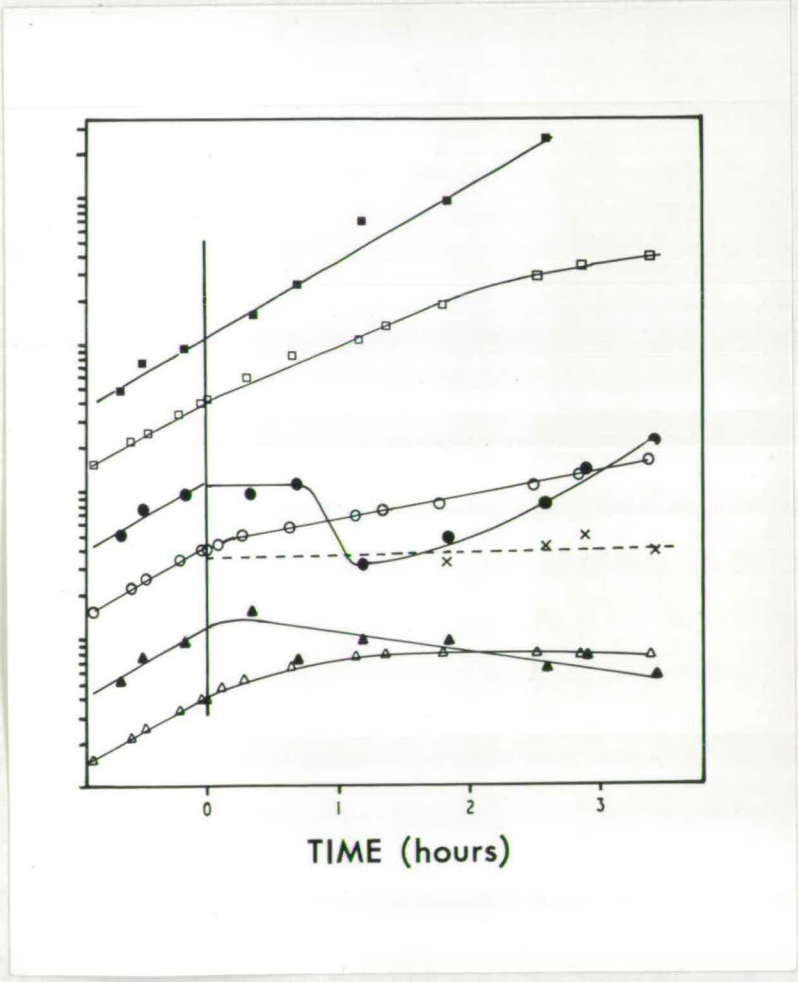
Fig. 21. Dose response curve for UV irradiation of W3110uvrA6. A culture of strain W3110uvrA6 was grown in 007 minimal medium to a density of 2 X 10 cells / ml. Penicillin was added to give a concentration of 10 IU / ml. Part of the culture was immediately diluted 1/10 into cold 007 salts (unsupplemented) with penicillin and irradiated. The required supplements were then added and the culture warmed to 37 C and grown for 1 mass doubling. The rest of the culture was grown for 1 generation in the presence of penicillin before being diluted into cold 007 salts and irradiated. Both cultures were then diluted and plated to assay viable cells on 007 medium plus 1%agar (Davis) in an overlay of 007 salts plus 0.7% agar. The plates were incubated for 48 hours at 37 C before counting. The numbers indicate the relative range in chromosome number of the two populations. Closed symbols, cultures that were irradiated after 1 generation of growth in penicillin; open symbols, cultures that were irradiated before 1 generation of growth in penicillin. The different symbols represent different experiments.



correct. The nature of the dose response shows that the lethal event is not the production of a simple lesion, such as a pyrimidine dimer, since at a dose of 40 ergs/mm^2 one would expect almost no survivors on this basis (this UV dose should produce about 250 pyrimidine dimers/chromosome: Howard-Flanders, 1968). This result is supported by the work of Yan (1969), who showed that cells growing on minimal medium with different carbon sources varied in their sensitivity to UV. Faster growing cells, which have a higher average DNA content, were more sensitive than slower growing cells. (This difference disappeared when the cells' ability to survive the decay of incorporated 32P was compared. This is not unexpected since 32P decay leads to production of single and double stranded breaks in the DNA, and thus the damaged chromosome would either be repaired or it would be rapidly degraded. In either case the 'blocked replicon' ceases to exist.)

The above interpretation of the basis of the dose-response curve for moi with P1 vs filamentation, and its correlation with the UV sensitivity of bacterial cells in relation to their DNA content, depends on two assumptions about the nature of the filaments induced by infection with damaged P1: i) that the growth rate of uninfected cells and filaments is the same, and ii) that the DNA replication of the host cell continues. Fig. 22 shows the growth of a culture infected with untreated P1 (bottom curves) or UVed P1 (top curves) at a moi of 16. From the time that filaments can be clearly distinguished in the culture infected with irradiated P1 their number remains constant, corresponding to about 25% of the population at the time of

Fig. 22. Growth of W3110uvrA6 infected with irradiated or untreated P1kc. A single culture was grown to a density of 3 X 10^7 cells/ml in LB. The culture was spun down and resuspended in 1/10 of the original growth medium. It was then split into 3 parts. One part (square symbols) was diluted back without any additions. The other parts were infected with P1kc irradiated with 3000 ergs/mm^2 of UV (circles) or with untreated P1kc (triangles) at an moi of 16 before being diluted back to the original cell density. Open symbols, A540; closed symbols, cell number (determined using the Petroff-Hausser counting chamber); crosses show the number of filaments / ml in the culture infected with irradiated P1kc. The vertical line denotes the time of addition of the phage.



infection. This figure is in keeping with the earlier suggestion that most of the input phage particles are inert, corresponding to an 'effective' moi of about 2.5, or about 1/6 of the input phage particles. Figs. 23 and 24 show the size distributions for these cultures at various times after infection. The proportion of the population that is of normal length, expressed as percent of total mass, does not increase significantly over the time period observed. It is also clear that in order for filaments of the length found to be produced, their length must increase exponentially at a rate very close to that of the control culture. During the period beween infection and the final size distribution shown, about 3 generations of growth would be expected on the basis of the growth rate of the control culture. Since the range in cell lengths at O time was 2-6, 3 generations of exponential growth in the absence of division should give a size range of 16-48, as compared to a range of 11-15 if growth was linear. The growth rate of the filamenting cells is the same as would be expected if they were growing normally (note: the cells infected with viable P1 do appear to grow at a constant rate).

Fig. 25 shows the effect of infection with normal or irradiated P1 on the rate of DNA synthesis. The rate of thymine incorporation is not reduced by infection with irradiated P1 for at least 3 generations after infection. It has previously been shown (Ikeda and Tomizawa, 1965; Beyersmann and Schuster, 1971) that host chromosome replication continues during normal lytic infection by P1 (a burst of DNA synthesis would not be expected in this experiment because of the high growth rate of the culture, which would be expected to mask the

Fig. 23. Samples taken at various times from the cultures shown in Fig. 22 were analysed for their size distribution as described in Materials and Methods. Ordinate, % of the total cells that fall within the size class; abcissa, cell length in arbitrary units (one unit is equal to approximately 1.3 μm).

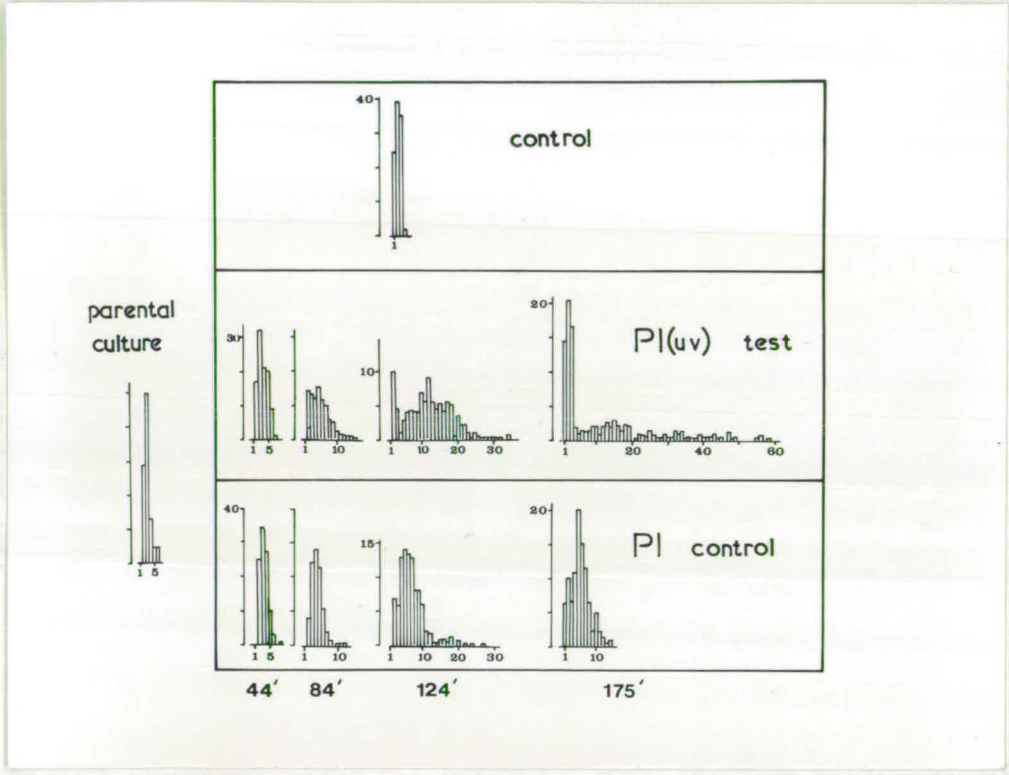


Fig. 24. Size distributions from Fig. 23 replotted to show the proportion of the total mass of the population that is represented by each size class.

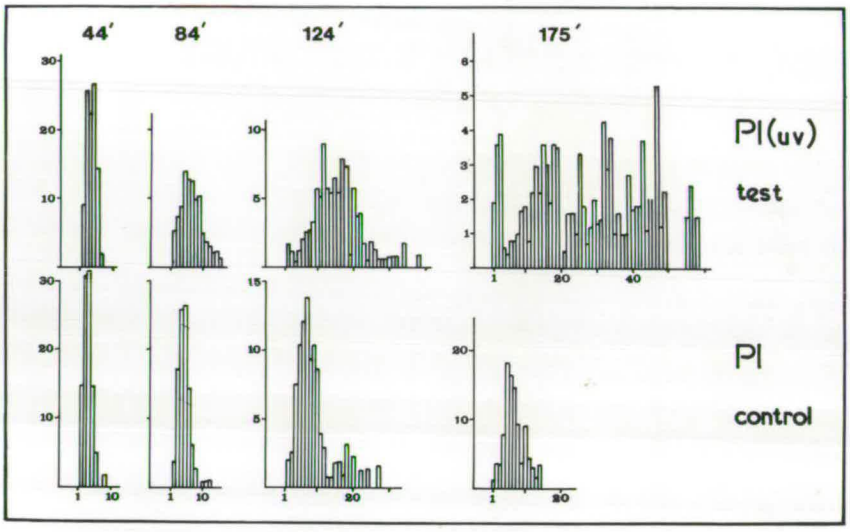
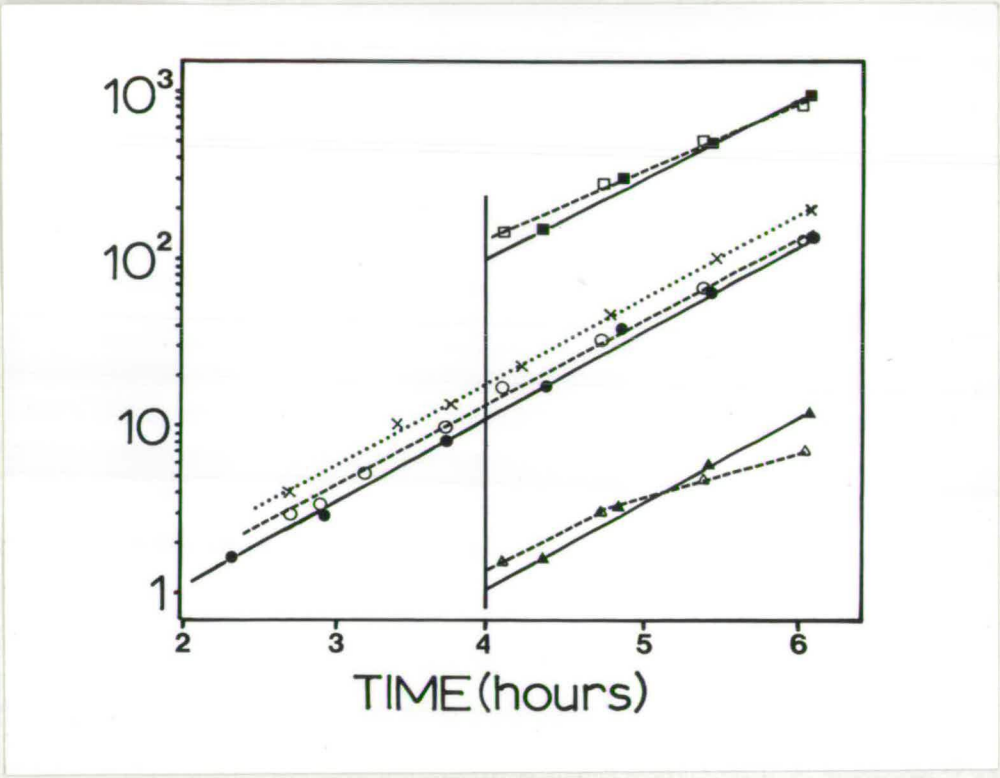


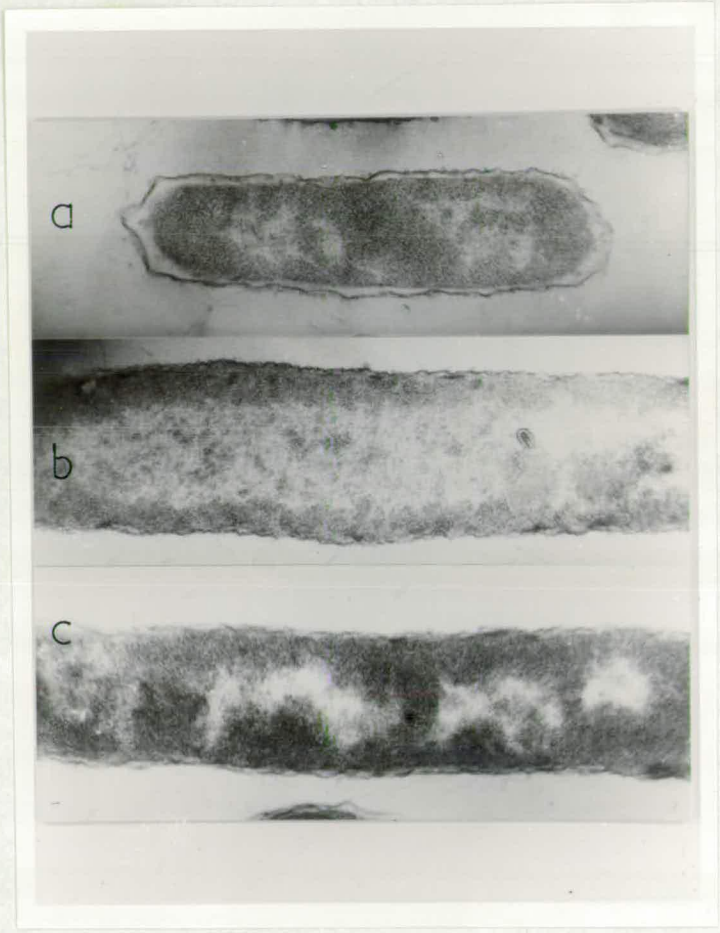
Fig. 25. A culture was treated as described in the legend to Fig. 22, except that the medium was supplemented with 10 uCi (methyl)-3H thymine / ml (specific activity approximately 0.22 Ci/mmol in the culture medium). Open symbols, A540; closed symbols, thymine incorporation measured as TCA precipitable counts. Circles, control culture; squares, cells infected with irradiated P1; triangles, cells infected with untreated P1; crosses, cell numbers in the control culture (determined using a Coulter Counter model A).



contribution of the phage DNA synthesis: cf. Scott and Schuster, 1973). Fig. 26 shows electron micrographs of thin sections of normal, P1 infected, and UVed P1 infected cells taken from the cultures shown in Fig. 25. It would appear that chromosome segregation also continues in the cells infected with irradiated P1. The contention that infection with irradiated P1 does not affect host chromosome replication is also supported by the continued exponential growth of the filaments. It seems unlikely that a cell could maintain an exponential rate of growth for more than 4 generations unless there was an increase in the cell's DNA content.

Fig. 26. Electron micrographs of thin sections of cells taken from the cultures shown in Fig. 25 120 minutes after infection with P1.

a. control culture
b. cells infected with untreated P1
c. cells infected with Irradiated P1.



iv). Discussion.

The experiments presented in this section suggest that a single damaged P1 genome can permanently block cell division. The fact that this block to cell division is permanent in a strain which is defective in repair but only temporary in a repair-proficient strain suggests that the blocking agent is the P1 DNA. The cells in which the block is permanent are unable to carry out normal excision of pyrimidine dimers (Howard-Flanders, Boyce, and Theriot, 1966). Thus while the infecting P1 genome could presumably circularize by recombination between its terminally redundant ends and initiate DNA replication, complete replication of the phage would not be possible. Similarly, at the level of UV irradiation used in these experiments it seems unlikely that functional phage proteins could be synthesized (a dose of 3000 ergs / mm^2 would be expected to introduce about 550 pyrimidine dimers into the P1 genome, as well as about 5 intrastrand dimers: Howard-Flanders, 1968).

Other replicons are also able to block cell division in this way, but not all replicons can do so. Thus F (Otsuji, personal communication), colI (Monk, 1969), and P1 can block cell division while phage lambda and Ø80 do not (unpublished observations). This classification follows that of the replicons that can bring about indirect induction of prophage lambda. Lambda is induced by the introduction of UVed F (Devoret and George,1967), colI (Monk, 1967,1969), RTF, and P1 (Rosner, Kass, and Yarmolinsky, 1968), but not by Hfr-donated chromosomal DNA (Devoret and George, 1967), or phages

434hy, T6, Ø80, f2 (Rosner et al, 1968), ØX174, or M13 (Monk, personal communication). It seems clear that both lambda induction and filamentation are the result, not of the presence in the cell of a piece of DNA containing UV photoproducts, but of a special kind of replicon that cannot complete a round of replication. The effective replicons are those which normally establish a stable relationship with the cell, maintaining about 1 copy of the episome/host chromosome but remaining physically independent.

The comparison of UV sensitivity of cultures with varying chromosome complements suggests that the bacterial cell can be killed by a lesion in only one of its chromosomes. Death in this case is most likely due to a block to cell division, and in keeping with the results with irradiated P1 a single block appears to stop division at all available sites. The implication of this finding for the nature of the coupling between DNA replication and cell division will be discussed in the next section.

C. A hypothesis for the nature of the interaction beween DNA replication and cell division.

In presenting a model for the way in which DNA replication might be coupled to cell division I hope to be able to pull together a number of separate lines of research that have been pursued in various laboratories. The model depends to a large extent on the apparent parallel between agents causing induction of prophage lambda and agents that block cell division by interfering with DNA replication. This parallel implies that both cell division and lambda induction share at some point a common step. This possibitity is reinforced by the isolation of a point mutation in E. coli that affects both processes. The mutant, T44, carries a temperature sensitive mutation. At the nonpermissive temperature a lambda lysogen is induced and a non-lysogen is blocked in cell division (Kirby, Jacob, and Goldthwaite, 1967). This mutation does not seem to affect DNA metabolism, and both effects are relieved by the recA mutation (Castellazzi, George, and Buttin, 1972).

For the sake of further argument, then, it will be assumed that information on the induction of lambda for which no parallel experiments on cell division have been or can be done is applicable to the argument.

First of all, lambda induction (but not vegetative replication) is dependent on the dnaA gene product* (Monk and Gross, 1971: this gene product is involved in the initiation of chromosomal DNA synthesis),

* in that λ is not induced in a dnaA mutant when DNA replication stops at the nonpermissive temperature

but dnaB, C, E, and G are not required (Schuster, Beyersmann, Mikolajczyk, and Schlicht, 1973). As a first hypothesis, then, I will assume that dnaA is involved in the coupling between chromosome replication and cell division. The lack of this gene product, however, is not sufficient to allow division to occur, at least not in normal temperature-sensitive dnaA strains. However there is a suggestion that such mutants can continue to divide in some cases (N. C. Jones, personal communication), and Hirota et al. (1969) have described a case where a secondary mutation, divA, allows such a strain to divide freely at the non-permissive temperature. As discussed by Schuster et al (1973) the role of the recA mutation in preventing lambda induction and filament formation could well lie in its interaction with the initiation process. The final point that should be noted is that lambda is induced in a dnaA mutant if residual DNA synthesis is temporarily blocked after the shift to the nonpermissive temperature or if the cell is irradiated with UV after the completion of residual DNA synthesis at the nonpermissive temperature (Monk and Gross, 1971).

A number of models have been proposed to account for the coupling of chromosome replication and cell division. However, if the results presented in the preceding section are taken into account, it becomes much more difficult to devise a hypothesis that will fit all of the experimental evidence. One possible hypothesis would be as follows.

The process of initiation of chromosome replication is coincident with a number of events in the cell cycle. A defined period of protein synthesis is initiated (see Donachie et al, 1973, for discussion), and

a new potential division site is defined (Donachie and Begg, 1970). The hypothesis then requires that whenever an initiation event occurs the eventual use of the associated potential division site is blocked by some means that requires the recA gene product. This block is duplicated by initiation on any replicon that requires the dnaA gene product. The termination of DNA replication and the termination of the fixed period of protein synthesis normally coincide (Pierucci and Helmstetter, 1969): one or more of the proteins synthesized in this period that are involved in septation could be involved in the induction of lambda if their normal site of action, the PDS, remains blocked. In a recA strain this block is never set up, so that cell division can procede in the absence of DNA replication (Inouye, 1971) and lambda induction does not occur. A reasonable candidate for the agent that releases the block to cell division would be the dnaA protein, released from the replication complex at the termination of replication. In a strain in which the dnaA protein does not function in initiation, the protein could still be capable of releasing the block on the potential division site so that lambda is not induced. However, if the release of the dnaA protein was delayed, by delaying the termination of DNA replication, until after the completion of the protein synthesis required for cell division, then lambda would be induced. One prediction of this model would be that a dnaC intiation mutant would not induce lambda at the nonpermissive temperature as quickly as would be the case if DNA replication was prevented by thymine starvation: a minimum of 40 minutes at the nonpermissive temperature would be required before infective centers were produced (the time required for newly initiated sequences of division protein

synthesis to be completed, at which point there would be no corresponding terminations of DNA replication). An even simpler test of this prediction would be to shift a lambda lysogen of a low-thymine requiring strain of E. coli to a medium containing a very low concentration of thymine. This treatment slows the replication velocity of the chromosome and thus lambda should be induced.

This model makes several other specific predictions about the cell cycle in E. coli that should not be difficult to test. For example, supernumary initiations on a chromosome might be expected to create additional blocks to the existing potential division site, so that division would be delayed until the latest round of replication had been completed. Such extra rounds of replication can be brought about (Worcel, 1970; Schwarz and Worcel, 1971; Kogoma and Lark, 1970). The best way to test this aspect of the hypothesis, however, would be the isolation of a new class of temperature sensitive mutants that initiate DNA replication in an uncontrolled manner at the nonpermissive temperature. Such cells would be expected to filament at the nonpermissive temperature and also to induce prophage lambda.

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Quantal Behavior of a Diffusible Factor Which Initiates Septum Formation at Potential Division Sites in *Escherichia coli*

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Analysis of nucleated cell size in a minicell-producing strain of *Escherichia coli* and in its parental strain shows that the two distributions are considerably different. A model is proposed to account for this difference. The model states that: (i) in the mutant population, the cell poles are available as potential division sites in addition to the normally located division sites; (ii) the probability of a division occurring at any of the potential division sites is equal; and (iii) only enough "division factor" arises at each unit cell doubling to permit a single division. This factor is utilized entirely in the formation of a single septum. Thus, the occurrence of a polar division with the production of an anucleate minicell (which occurs only in the mutant strain) prevents the occurrence of a non-polar division, with the result that the average nucleated cell length is increased in minicell-producing strains. The model has been used to construct a theoretical population, and a number of parameters of the real and theoretical populations have been compared. The two populations are very similar in all of the parameters measured.

In the normal cell cycle of *Escherichia coli*, the cell volume doubles, the cylindrical cells doubling in length without any apparent change in diameter. The cells then divide in the middle of the long axis. Donachie and Begg (6) have shown that a potential division site, detected by its sensitivity to penicillin, is formed very early in the cell cycle. The number of potential sites increases at each doubling of a constant unit cell volume.

In this paper we present an analysis of the pattern of cell growth and division in *E. coli* strain K-12 P678-54, a mutant strain isolated by Adler and Hardigree (1). In this strain, cell division can take place not only in the normal way, by septation between two nucleated regions in the interior of the cylinder, but it can also take place very close to the cell poles. If division takes place at a polar site, a very small, anucleate "minicell" is cut off from the parent cell (Fig. 1). In the wild-type strain, each potential division site is used only once, giving rise to two cell poles. The mutant strain behaves as if potential division sites, once formed, are never inactivated. Hence, the cell poles, which represent the sites of preceding divisions, can serve as sites for further divisions, giving rise to minicells.

MATERIALS AND METHODS

E. coli K-12 P678 (Thr⁻Leu⁻Thi⁻) and the minicell-producing derivative P678-54 were used in these experiments. Minicell production has been reported to be the result of two mutations at different loci (K. Roozen and R. Curtiss III, Abstract no. 4607A of Oak Ridge National Laboratory, 1969). The genotype of P678-54 is therefore Thr⁻Leu⁻Thi⁻MinA⁻MinB⁻. Both strains were obtained from H. Adler.

Media and experimental techniques. Cells were grown at 37 C in either L broth (10 g of tryptone [Difco], 5 g of yeast extract [Difco], and 10 g of NaCl in 1 liter) or minimal medium (007 salts [3] plus 0.2% glucose [wt/vol], 50 μg of threonine per ml, 50 μg of leucine per ml, and 10 μg of thiamine per ml). The generation time for both strains was 26 min in L broth and 65 min in minimal medium.

The distribution of cell lengths in exponentially growing populations was obtained by spreading the cells on thin layers of 1.2% agar containing 0.05% sodium azide, and photographing the cells with a Zeiss Ultraphot microscope by using phase contrast. In some experiments, observations were made on living cells growing on a thin layer of 1.2% agar containing growth medium. Measurements were made on enlarged projections of the negatives.

For electron microscopy, cultures (10 ml) were grown in exponential phase to approximately 5×10^7 cells/ml, fixed with glutaraldehyde (10), and centrifuged at 13,000 × *g* for 30 min. The cells were

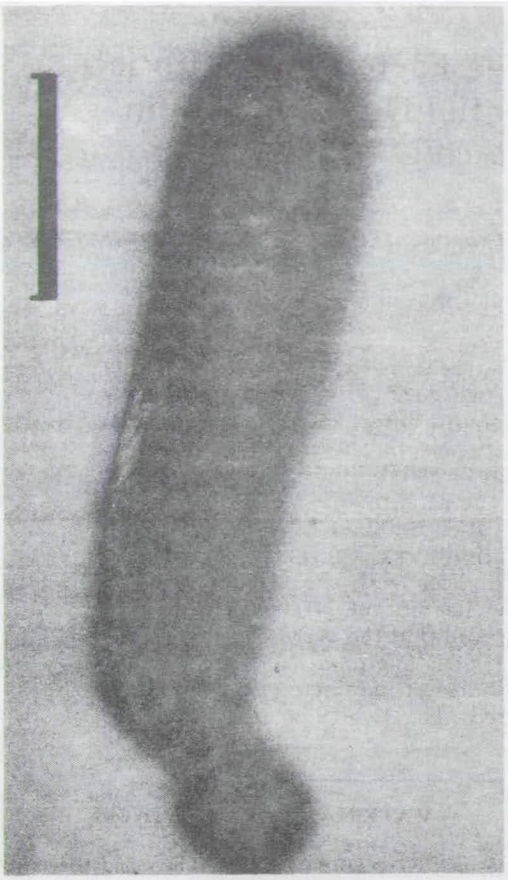

FIG. 1. *Polar division of strain P678-54. Cells were grown at 37 C in L broth, fixed with glutaraldehyde, washed, placed on grids, and stained with uranyl acetate. The photographs were taken with a Siemens Elmiskop 1A electron microscope. Bar equals 1 μm.*

washed twice with 10 ml of 1% ammonium acetate and resuspended in 0.5 ml of 1% ammonium acetate. Samples were placed on freshly prepared grids and stained with uranyl acetate (10). Photographs were taken with a Siemens Elmiskop 1A electron microscope.

RESULTS

The length distribution of 350 cells from an exponential population of wild-type strain P678 is shown in Fig. 2a. Over 95% of the cells lie within a twofold range. This corresponds very well with the size distribution expected from an ideal population in exponential growth.

The cell length distribution of minicell-producing strain P678-54 under the same growth conditions is shown in Fig. 2b. In this case, the range in size of a sample of 400 cells is at least ninefold. The presence of long cells in populations of P678-54 has been reported previously (1). The minimal length for cells is approximately the same for both strains (excluding minicells from the measurements), but the average cell length of P678-54 is 1.68 times that of P678. Cells of both strains grown in minimal medium were smaller, but the relative range of cell size and the shape of the length distributions were unchanged.

Two other minicell-producing strains isolated in this laboratory (Teather, unpublished observations) show a similar proportion of long cells in the population.

A model was devised to account for the length distributions shown in Fig. 2. The assumptions of the model are as follows. (i) Potential sites for cell division arise during cell growth in the same way in both strains. Such sites are active for only one division in strain P678, but they remain active indefinitely in strain P678-54. A division site at which a division has occurred forms two cell poles. In strain P678-54, subsequent divisions can occur at these sites and will produce minicells. (ii) The probability of division is equal at all potential division sites, whether polar or nonpolar. (iii) If a newborn cell in an exponentially growing wild-type popula-

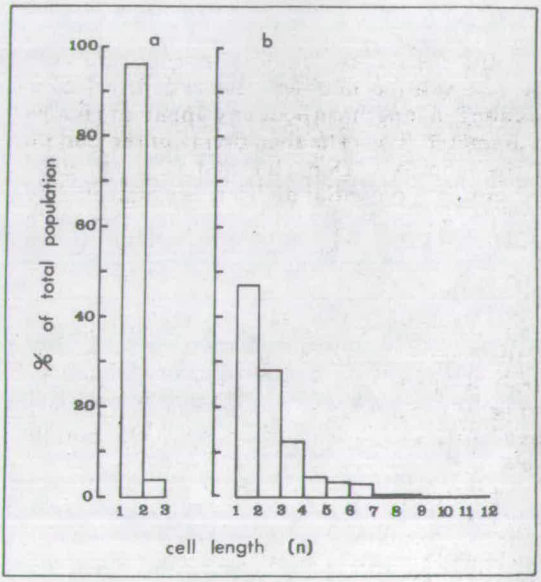

FIG. 2. *Size distributions of strains P678 (a) and P678-54 (b). Cells were grown in L broth, spread on a thin layer of agar containing 0.05% sodium azide, and photographed in a Zeiss Ultraphot photomicroscope using phase contrast. Measurements were made on enlarged projections from the negatives. The size classes were then grouped in multiples (n) of the minimal length (c) of strain P678.*

tion has the length c, then division will not occur until a length of $2c$ is reached. At this size, there is only enough of some specific factor (division factor) for a single division. This division factor is consumed entirely in the formation of a single septum. If there is more than one division site available (as in the minicell-producing strain), the division factor is expressed solely at one of the available sites.

As a consequence, the length of a daughter cell in a minicell-producing strain may be a multiple of the newborn cell size (c) of a wild-type strain. If the length of a particular newborn cell is $n \cdot c$ (where n is an integer), the cell after one doubling in mass will be $2n \cdot c$ in length and will have produced sufficient division factor to undergo n divisions. The number of available division sites will be the normal number of internal division sites ($2n - 1$) plus the two cell poles, giving $2n + 1$ division sites.

Using these assumptions, it is possible to predict the length distribution of a minicell-producing population (Table 1). For any newborn cell of length $n \cdot c$., the table shows the expectation of producing a daughter cell of a particular length one mass doubling later. (The mathematical procedures used to generate these expectations are discussed in the appendix to this paper.)

These expectations have been used to predict the length distribution of an exponentially growing population of cells. The length distribution generated in this way is shown in Fig. 3a. Figure 3b shows the observed and predicted distributions after the predicted distribution was altered by shifting 10% of each size class to each adjacent size class. This transformation of the theoretical distribution was carried out because there is a significant probability of classification errors in the observed distribution when the length of the projected image is within 1 mm of a class boundary. The χ^2 value (10 degrees of freedom) was 3.5, indicating a satisfactory measure of agreement ($P > 95\%$).

The length distribution of the progeny of any cell should approach the predicted distribution within a few generations, even if the initial cell was abnormally long. To test this, individual cells growing on L-broth agar slides were observed and photographed at frequent intervals. Cells at least two times the average length were

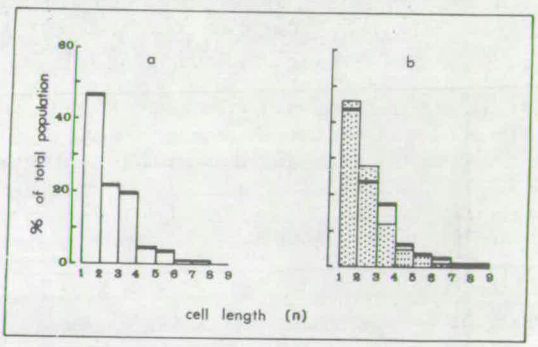

FIG. 3. *Theoretical size distribution according to the model. (a) Predicted distribution. (b) Predicted distribution after "smearing" to allow for probable experimental classification errors (heavy bar). Smearing was accomplished by shifting 10% of the total in each size class to each adjacent size class. Superimposed on this distribution is the observed distribution for strain P678-54 (shaded area).*

TABLE 1. *Predicted pattern of daughter cell lengths*

Newborn cell size	Properties of the cell at division			Relative frequency of newborn cells of sizes shown[a]								
	Size	No. of division sites available	Division factors available	Mini	1c	2c	3c	4c	5c	6c	7c	8c
nc	2nc	$2n + 1$	n									
1c	2c	3	1	0.67	0.67	0.67						
2c	4c	5	2	0.80	1.00	0.70	0.40	0.10				
3c	6c	7	3	0.86	1.43	0.91	0.51	0.23	0.06			
4c	8c	9	4	0.89	1.89	1.15	0.63	0.30	0.11	0.02		
5c	10c	11	5	0.91	2.36	1.39	0.76	0.37	0.15	0.05	0.01	
6c	12c	13	6	0.92	2.85	1.64	0.88	0.43	0.19	0.07	0.02	
7c	14c	15	7	0.93	3.33	1.89	1.01	0.50	0.22	0.09	0.03	0.01
8c	16c	17	8	0.94	3.82	2.14	1.13	0.56	0.26	0.10	0.04	0.01

[a] The expectations given represent the relative frequency with which parental cells, when they divide, will give rise to daughter cells of the sizes shown. The sum of the expectations in any given case is equal to $n + 1$, the number of daughter cells produced from one cell. For example, a normal *E. coli* cell, dividing at length $2c$, gives rise to two similar daughter cells of length c. The expectation for cells of length c arising is therefore 2.

selected for this analysis. Figure 4 shows an example of a life history of such a cell (initial length, 5c) and its progeny. Because the predicted distribution applies only to exponentially growing cells, the rate of growth of individual clones was measured. Figure 5 shows that the growth of the cells under such conditions is in fact exponential. A combined size distribution for the progeny of these large cells after two to three generations is shown in Fig. 6, superimposed on the predicted distribution from Fig. 3b. The value of χ^2 (10 degrees of freedom) was 3.0, again indicating a satisfactory measure of agreement ($P > 97\%$).

Other predictions of the model were tested. An exponentially growing population of wild-type cells which range in length from c to $2c$, where c represents the normal newborn cell size, should have a mean length of $1.44c$ (12). If the normal, minimal, newborn cell length in P678-54 is also c under the same growth conditions, then the predicted mean cell length for P678-54 is $2.53c$ (see Appendix for details of these calculations). The mean lengths observed for P678 and P678-54 were $1.46c$ and $2.46c$, respectively.

Another prediction of the model is that there should be a constant ratio (if the minicells are stable) of 0.75 minicells per nucleated cell in an exponential population (see Appendix). A direct count of minicells, with an electron microscope, gave a ratio of 0.67 ± 0.08. This method may possibly underestimate the proportion of minicells; first, because minicells may be preferentially lost during preparation for electron microscopy and, second, because the length of time for which minicells persist after formation is unknown. Measurement of the relative frequencies of polar and nonpolar divisions was therefore made by microscope observation of living cells growing on nutrient agar. The ratio obtained by this method was 0.72 ± 0.11.

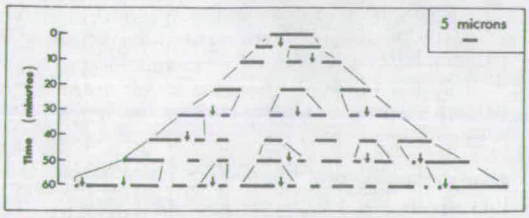

FIG. 4. *Growth and division of a large cell of strain P678-54 growing on L broth plus 1.5% agar (Davis) at 37 C. Arrows show divisions that have occurred since the previous observation. The growing cells were photographed by phase contrast, and measurements were made on enlarged projections of the negatives.*

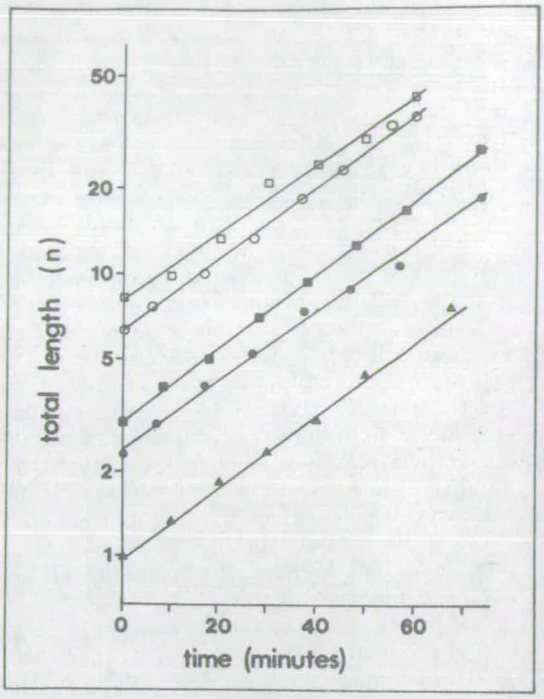

FIG. 5. *Growth of strain P678-54 on L broth plus 1.5% agar (Davis). Each symbol represents one cell (zero time) and its progenies. The log of the sum of the length of all cells in a clone (ordinate) is plotted against time. Measurements were made as described in the legend to Fig. 4. Growth is clearly exponential.*

Among cells which divide at a length of $2c$, the predicted frequency of polar divisions is 0.67. Direct observation of cells of this length growing on nutrient agar gave a frequency of polar division of 0.61 ± 0.08. Minicells were also observed to be produced at equal frequency from both "new" and "old" cell poles, confirming another assumption of the model.

DISCUSSION

It can be concluded from the observations made that our model provides an accurate description of the growth and division of this minicell-producing mutant. An effort was made to devise alternative models consistent with the known biology of *E. coli* that would fit the experimental data. For example, if the location of division sites were random within a cell, then cells of all lengths would be produced, including both very long and very short cells. This possibility can be excluded because no cells intermediate in length between minicells and the normal newborn cell are found in the mutant population. (Although this model therefore clearly does not apply to P678-54, mutants

of this kind may exist and would appear superficially similar to this strain.) Another model which might be considered states that the amount of division factor produced at each mass doubling is equal to the number of nonpolar, potential division sites. In this case, the predicted average cell size would be much less than that observed in P678-54. Other models which we considered initially have also been ruled out by the experimental findings reported here.

The pattern of division observed in the mutant suggests that the substance responsible for triggering division at any given site behaves as a quantum. Specific cell histories can be used to show that this factor could not be distributed in association with, or as a consequence of, localized cell envelope growth. Figure 7 shows one such history that has been observed. The "division factor" therefore behaves as a diffusible entity.

The nature of this diffusible substance which is responsible for triggering division is unknown. However, termination of a round of chromosome replication is known to be required for each cell division (2, 11). It also seems possible that the chromosome can "diffuse" within the cell (5, 13). It is therefore possible that the attachment and replication of the chromosome at a potential division site is required for division at that site after replication. However, the triggering event might not be termination itself but the attendant production of some diffusible substance, such as the "termination protein," described elsewhere (7, 9). In this case, the location of the replicating chromosome(s) within the cell would not be a factor in the determination of the localization of the next division.

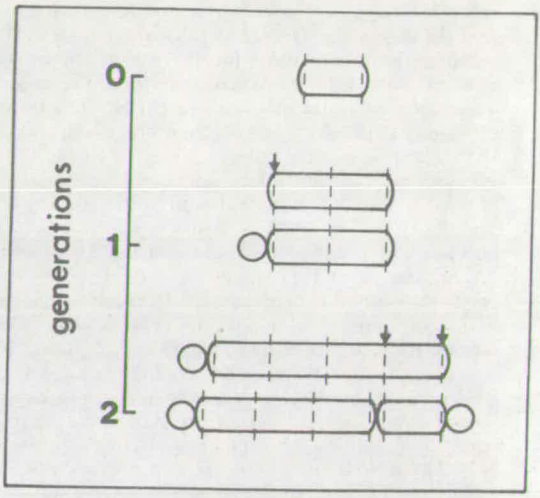

FIG. 7. *One observed division pattern for strain P678-54 growing on L broth plus 1.5% agar. Dotted lines show the presumed location of potential division sites. Arrows show the sites at which division is initiated.*

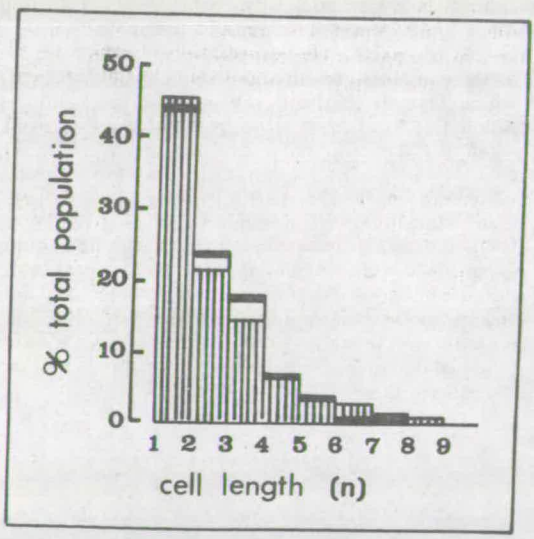

FIG. 6. *Size distribution for the progeny of large cells ($n \geq 5$) of strain P678-54 after two to three generations of growth on L broth plus 1.5% agar (Davis) at 37 C (shaded area). Superimposed on this distribution is the predicted distribution from Fig. 3b. Measurements were made as described in the legend to Fig. 4.*

The model proposed contains only one assumption that is specific for the mutant strain: that potential division sites persist. The other statements apply equally well to the mutant and to normal strains. If the model is correct, then in *E. coli* there are distinct processes controlling the localization and the number of cell divisions.

APPENDIX

Normal and abnormal cell length distributions. The distribution of cell lengths observed in an exponentially growing population of rod-shaped organisms depends primarily on two characteristics: (i) the distribution of the cell lengths at birth and (ii) the pattern of length increase during the cell cycle (4). The parental strain of *E. coli* P678 shows an almost ideal length distribution consistent with cells born at a single length and growing linearly with time until they divide at the end of the cell cycle. The mean cell size (see above) is very close to the theoretical value of 1.44 times the newborn cell size (12).

Interpretation of cell length distributions in mutants of increased average length can help reveal the physiological basis of the mutation. For instance, if there were an increase in the size of the newborn cell, the length distribution should resemble the parental

one, drawn on a larger scale, and should retain the same relationship between the newborn cell size (c) and the mean size ($1.44c$) as the parental strain.

An alternative cause for increased length in a mutant could be the occasional failure of division to occur at a potential division site (PDS). The relative efficiency with which the division process occurs at a PDS and the increased size are related in the following manner. Consider a large number of organisms, N, of total length, L, of the parental strain, in which we assume that every PDS is used when formed with an efficiency of 1. After one doubling time, there will be $2N$ organisms of total length $2L$. N divisions have occurred, and the necessary N PDS were provided with the increase in length, L; that is, one PDS is formed for every increase in length of L/N units. Now consider the mutant strain; N organisms have a total length kL, where k is the observed average increase in length over the parental strain. After one doubling time, there will be $2N$ organisms with a total length $2kL$. The length increase is kL, and kN new PDS will have been formed. In addition, there were $(k - 1)N$ unused PDS present in the original organisms, equal to the total original number present (kN) less those already used in forming the N organisms (N). The N new divisions are distributed among the unused sites, which total $(2k - 1)N$. The probability of a division at an unused site is therefore $N/(2k - 1)N$, or $1/(2k - 1)$. The proportion, p, of used to total sites remains constant at N/kN, or $1/k$. If the distribution of the used sites among the total sites along a hypothetical chain of organisms is random, the probability that n segments between adjacent PDS are included in a new organism is $h(n)$, where

$$h(n) = (1 - p)^{n-1}p \qquad \text{which reduces to}$$

$$h(n) = (k - 1)^{n-1}k^{-n}$$

The length of the segment between adjacent PDS is constant in newborn cells (6, 7). The observed population in each length class will consist of cells which may have been born at different sizes and are thus at different stages of the cell cycle. In a stable, exponentially growing population of organisms born at length a, dividing at length $2a$, and increasing in length linearly, the fraction of the population of length l is $2^{(2 - l/a)} \cdot \ln(2)/a$ (12). The proportion of the population between lengths a and l is

$$\int_a^l 2^{(2 - l/a)} \cdot \ln(2)/a \cdot dl = 2(1 - 2^{(1 - l/a)})$$

The observed population of length less than or equal to l, $Pop(l)$, can be expressed as the sum of the contributions corresponding to each birth size (nc) in proportion to the frequency of such births, $h(n)$.

$$Pop(l) = \sum_{n=1}^{n=\infty} h(n) \cdot 2(1 - 2^{(1 - b/nc)}) \qquad (1)$$

where the variable $b = 2nc$ when $2nc \leq l$, and $b = 1$ when $2nc > l$. The fraction of the population lying between lengths l and $l + x$ is calculated as the difference between $Pop(l + x)$ and $Pop(l)$.

Such a model does not, however, include any statement about the production of minicells by polar division. The simplest model that combines the modified division property with the occurrence of minicells among the population assumes that the synthesis of each new unit of cell length c leads to the accumulation of one "division factor" which has an equal probability of being used at any PDS, whether internal or at either pole; polar septation produces a minicell. For each initial cell size, there will be a variety of possible patterns for the ensuing division process. The distribution of progeny sizes and the rate of minicell production can be calculated from the possible arrangements into groups of indistinguishable particles which have Bose-Einstein statistical characteristics (8). An organism of initial length nc will grow to length $2nc$, and n division factors will be formed. These will be distributed among the $(2n - 1)$ internal PDS and two polar PDS. n septa will be formed in the dividing organism, and $(n + 1)$ progeny will be produced. Septation is possible at either or both poles, but at least $(n - 2)$ septations must occur at internal PDS, which will result in $(n - 1)$ organisms each of minimal length c. $(n + 1)$ units of length c remain to be distributed among the progeny in a random fashion, and all the possible distributions define all the possible ways in which the dividing organism can complete n septations. The probability of any of the progeny receiving x additional units of length c is $p(x)$, where:

$$p(x) = \frac{(2n - x)!\,(n + 1)!\,n!}{(2n + 1)!\,(n + 1 - x)!\,(n - 1)!}$$

(see reference 8, p. 59, equation 5.1). The addition of any units of length c to either of the two potential minicell progenies results in nonappearance of the minicell and formation of an additional viable organism in its place. The expectation of progenies of length ac arising from an organism of initial length nc at the end of its growth and division cycle is $Pr(n,a)$, where $Pr(n,0) = 2p(0)$ (the probability of minicell production) and $Pr(n,a) = 2p(a) + (n - 1)p(a - 1)$ when $a \neq 0$. Computed values are given in Table 1. Since the progenies from any one size of initial cell are varied in length, the distribution of newborn organisms that maintains a stable length distribution as the population grows (an essential feature of a stable exponentially growing population [4]) is such that the number of progenies formed at any size after one generation is twice the number of newborn organisms of that size initially present. If the frequency of newborn organisms of length nc is $f(n)$, and of length mc is $f(m)$, then

$$\sum_{n=1}^{n=\infty} f(n) \cdot Pr(n,m) = 2f(m)$$

The values of $f(n)$ were computed by simulating the growth of a single organism of length c through many generations till the relative proportions of each size class stabilized. This distribution of initial cell sizes was converted into the length distribution that should be observed in an exponentially growing population (Fig. 3a) by equation 1, substituting $f(n)$ for $h(n)$. The average length of the population is $2.52\ c$, where c is

the smallest newborn cell size, and minicells are produced in the ratio 0.748 per cell per doubling time. If the minicells are stable, this ratio should be constant.

A variant of this model in which only one end of a dividing organism retains the capacity to undergo a division process with the formation of a minicell has been similarly analyzed. An organism of initial length nc doubles in length to $2nc$, and then has the capacity for n divisions. These will form n septa, giving $(n + 1)$ progenies. Septation is possible at one pole only, so at least $(n - 1)$ septa must be formed at internal PDS, producing n organisms of minimal length c. n units of length c remain to be distributed among the progenies in a random fashion. The probability of any of the progenies receiving x additional units is given by $p(x) = [(2n - x - 1)!\ n!\ n!]/[(n - x)!\ (n - 1)!\ 2n!]$. The expectation of new organisms of length ac arising from the divison after doubling of an organism originally nc units long is $Pr(n,a)$ where

$$Pr(n,0) = p(0) \qquad \text{(the rate of minicell production)}$$

and

$$Pr(n,a) = p(a) + n \cdot p(a - 1)$$

when $a \neq 0$. The average initial cell length is 1.5 c, and the mean observed cell length in an exponentially growing population is 2.16 c. Minicells will be generated at a rate 0.5 per organism per doubling time. Both the mean size and minicell production level distinguish this model from the previous one in which both ends of the organism were possible sites of minicell production, although direct observation of division in cells of various sizes can provide a more direct test.

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THE BACTERIAL CELL CYCLE

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INTRODUCTION

e growth and development of all higher animals and plants involves
growth and division of their component cells. This growth and
ision may be accompanied by progressive cell differentiation as the
tial cell line develops into the various tissues that comprise the whole
ganism. Nevertheless, since the growth and division of single cells is a
mmon denominator in all such developmental sequences, it is appro-
ate to include studies of the cell cycle itself in any general considera-
n of differentiation.

In addition to the fact that the cell cycle is an obligatory component
most embryological development, it is also true that the growth of a
gle cell, from its creation at the division of its parent through the steps
uired to enable it to divide in its turn, is a process of differentiation.
e newly formed cell differs from a cell about to divide not only in size
t, as we shall see, in its composition, and this composition changes
alitatively in a fixed sequence throughout the cell cycle.

In general the process of cellular differentiation can be studied more
ily in bacteria than in higher cells. *Escherichia coli* is probably the
st understood organism in terms of its molecular biology and this
kes the investigation of the cell cycle easier than in organisms where
nature of fundamental molecular processes, such as the regulation of
ne activity, is still ill understood. We believe therefore that the
estigation of the cell cycle in *E. coli* and other bacteria should be able
proceed much more rapidly than in other organisms. Although this
ocess may prove to differ in some ways from that of higher cells (as
obviously must when the relative simplicity of the bacterial cell is
nsidered) it will probably, like earlier work on the molecular biology
bacteria, prove illuminating and helpful to the investigation of
karyotic systems.

THE DNA REPLICATION CYCLE

The nature of chromosome replication in Escherichia coli

The genome of *E. coli* consists of a single closed circle of DNA. T circle, about 1200 μm in circumference, has been the object of intens study, both genetical and biochemical. Nevertheless, probably no m than 10 % has so far been identified with specific genetic functions (Taylor, 1970) and the biochemical mechanism of its replication is s not understood (see Gross, 1972, for review). Even less is known ab the spatial and temporal organisation of this enormous molecule wit a cell which is itself only about 2 μm in length.

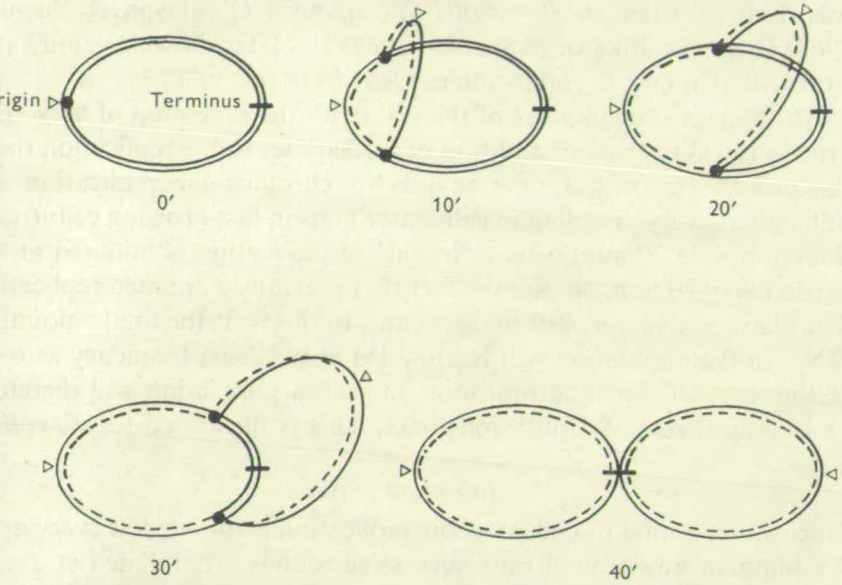
On one level, however, the control of the replication of this genome c be fully described. This is in terms of the way its replication is integra into the bacterial cell cycle.

Replication begins on the bacterial chromosome at a fixed site ('origin') and proceeds in both directions from that point until the t replication forks reach a point (the 'terminus') which appears to located on the circle approximately opposite to the origin (Masters Broda, 1971; Bird, Louarn, Martuscelli & Caro, 1972; McKenna Masters, in press). This process is shown in Fig. 1.

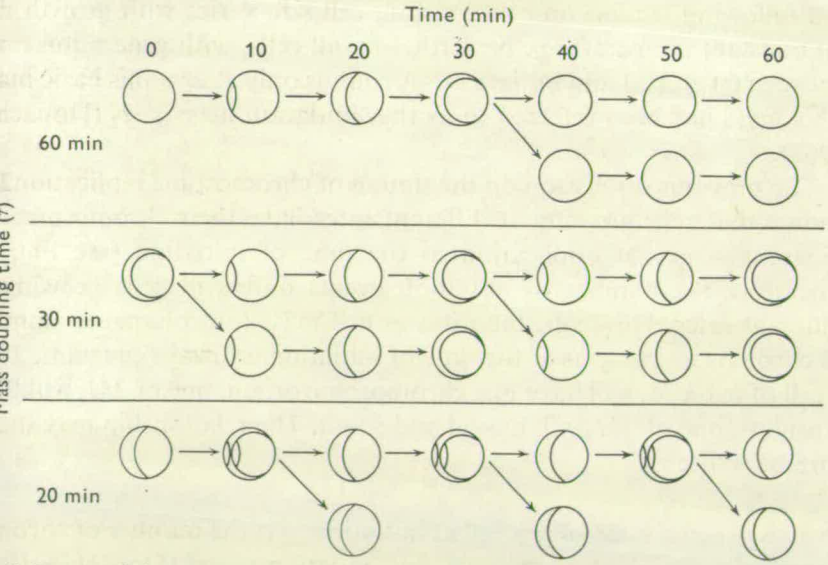
The time required to duplicate the genome is therefore limited by rate at which nucleotides can be added to the growing daughter stra at the two replication forks. As Cairns first showed, this process is sl relative to the rate of growth of the bacterial cell itself (Cairns, 19 In fact the complete sequential duplication of this molecule, from ori to terminus, takes approximately 40 min (at 37°) under a wide vari of growth conditions, although the length of time required to dou the rest of the components of the cells can range from 60 min to as li as 20 min under these same conditions (Clark & Maaløe, 19 Helmstetter, 1967, 1968, 1969; Cooper & Helmstetter, 1968).

Control of the timing of initiation

This slow rate of movement of the replication forks around the chro some therefore results in the curious situation that the time taken duplicate a chromosome may be twice as long as the cell cycle itself conditions where cells are growing rapidly (in a rich growth mediu Nevertheless, such fast-growing cells are able to divide every 20 min each daughter cell receives a complete genome. This is achieved b simple control system which appears to be identical in the two distan related species in which it has been studied, *E. coli* (a gram-nega organism) and *Bacillus subtilis* (a gram-positive organism). This sys



. 1. Diagrammatic representation of the replication of the genome of *E. coli*. DNA lication begins at a fixed site (the origin) on the 'chromosome' (actually a topologically ular double helix of DNA, here drawn as two parallel lines representing the two single nds of the helix). The black dots represent the positions of the replication complexes. wly synthesised strands of DNA are shown as dotted lines. Replication proceeds at a stant rate until the two replication forks meet at the opposite side from the origin. This nt, the terminus, is shown as a bar. The whole process takes about 40 min (in most wth media at 37 °C).



. 2. Pattern of chromosome replication over a 60 min period in cells growing at different s. The double helix is represented this time as a single line. In every case, each pair of ication forks takes 40 min to travel around the chromosome from the origin to the minus. A new round of replication is initiated at intervals equal to the mass doubling e of the cells, whether or not the previous round has been completed.

was first described in *B. subtilis* (Yoshikawa, O'Sullivan & Sueok 1964; Oishi, Yoshikawa & Sueoka, 1964) and later shown to apply al to *E. coli* (Cooper & Helmstetter, 1968; Masters, 1970).

At different growth rates of the cell (with the exception of very sl growth rates) the rate of addition of nucleotides to the replication fo remains the same but new rounds of chromosome replication a initiated at every doubling in cell mass. Thus in fast-growing cells (m doubling time 20 min) a new 'round' of replication is initiated at t origin every 20 min, despite the fact that previously initiated replicati forks have not yet reached the terminus. In this way, the total amount DNA in the population will be doubled at the same frequency as to cell mass itself. Each chromosome in such a population will therefo have several sets of replication forks. This is illustrated for *E. coli* Fig. 2.

Initiation mass

Since a new round of chromosome replication is initiated at every m doubling, at any growth rate, successive rounds are initiated at 2^n. (where n is any integer and M_i is the mass of the cell at the first initiati event). This introduces the question of the value of M_i under differ growth conditions. Surprisingly, it turns out that this value is const (at least to a first approximation) over a wide variety of growth con tions. This value appears to be equal to the mass of a newly divided c growing with a generation time (τ) of 60 min. As will be explained the following section on cell division, cell size varies with growth r (at constant temperature). Nevertheless, all cells, with generation tim between 60 and 20 min initiate DNA rounds only at $2^n \times$ this basic ma This mass has been referred to as the 'initiation mass', M_i (Donach 1968).

The previous discussion on the timing of chromosome replication h shown that cells growing at different rates have their chromosomes different stages of duplication at the time of initiation (see Fig. Therefore the number of initiation events differs in cells growing different rates. However, the ratio of cell mass (or volume) to numb of chromosome origins at the time of initiation is always constant. Th a cell of mass M_i will have one chromosome origin, one of $2M_i$ will ha 2 origins, one of $4M_i$ will have 4 and so on. The relationship may the fore be written:

$$m/o = M_i$$

(where m is the mass of the cell at initiation, o is the number of chrom some origins and M_i is the constant initiation mass) (Donachie, 19 Donachie & Masters, 1969).

Exceptions

e simple rules for the control of DNA replication in *E. coli* do not pear to hold under conditions of very slow growth in poor media ooper & Helmstetter, 1968). At doubling times greater than about min, the time from initiation to termination (C) lengthens (to 3τ) and initiation may take place at a cell mass less than M_i (Helmtter, personal communication).

In certain DNA mutants also, initiation may take place at cell masses ferent from $2^n . M_i$ (Worcel, 1970).

These exceptions need occasion no surprise; it is the constancy of the ationship $m/o = M_i$ under so many growth conditions which is rprising. The explanation of this relationship is not known at this ne but it would seem unlikely that cell mass *per se* is the signal for initian. Two main kinds of mechanism have been suggested. One is that eplication complex is built up, perhaps at a specific location on the l surface, at a rate proportional to overall growth rate and that this is ature' when a certain amount of cell growth has taken place. Initian then takes place by the combination of the chromosome origin th the replication complex (Donachie, 1968; Helmstetter, Cooper, rucci & Revelas, 1968; Bleecken, 1971). Two new replication mplexes would then start to be made at the same time as the first mplex matured. An alternative model has been suggested by Pritchard, rth & Collins (1969) who suggest that an inhibitor of initiation could made over a short interval immediately following the beginning of romosome replication and that further increase in cell volume would required to dilute this inhibitor below a critical threshold. However, -one yet knows how initiation is controlled.

CONTROL OF CELL DIVISION

The timing of cell division

e process of cell division must also be related to this process of romosome replication, if each newly formed cell is to receive a copy the genome. The mechanism by which this is achieved is still unown but the timing of division can be accurately predicted under ost growth conditions since cell division normally takes place out 20 min after the completion of each round of chromosome lication (Cooper & Helmstetter, 1968). It is clear therefore that l division does not take place at any particular cell size and, in ct, one consequence of this system of regulation is that cell size

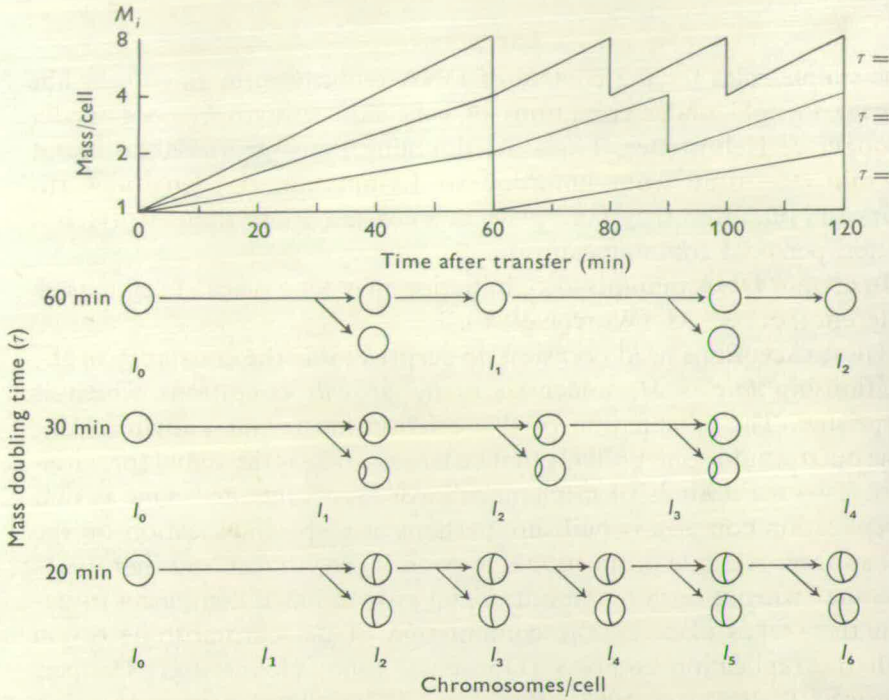


Fig. 3. The timing of cell division and of rounds of chromosome replication. In this hy thetical experiment three single cells, each of mass $1 \cdot M_i$, are inoculated into three diffe media, in which the mass doubling times are 60, 30 and 20 min respectively. In each case first division takes place 60 min after inoculation into the new medium. In consequ cell size at this first division is a function of the growth rate in the particular medium. fact, log (mass/cell) $= k/\tau$.) Each successive division takes place 60 min after each succes doubling of the initial unit cell mass (M_i). After the first division, therefore, the inte between successive divisions is equal to the mass doubling time.

At 0 min, each cell has a single unreplicated chromosome and starts replicatin immediately. Successive rounds of chromosome replication are initiated at each succes doubling of the unit cell mass (M_i). I_0, ... I_n, represent the times of successive initiation the three cell lines.

must increase with increase in growth rate. The way in which this con about is illustrated in Fig. 3. In this figure, a cell which has been growi with a generation time of 60 min is transferred, immediately af division (i.e. at mass M_i), to one of three different growth media. T mass doubling times in these media are 60 min, 30 min and 20 m respectively. (These different growth rates may be attained by addi various extra nutrients to the basic salt glycerol growth medium.) cells growing with a 60 min doubling time, initiation of chromoso replication takes place immediately after division. To construct Fig (which is diagrammatic and approximate), it is assumed that grow rates change immediately on transfer to the new medium, that initiati

kes place immediately on transfer to the new medium, that initiation kes place at every mass doubling thereafter and that cell division takes ıce 20 min after every termination. It can be seen that, as a conse- ence, cell size at division (or average cell size) is four times greater ıen the doubling time is 20 min than when it is 60 min. Such a fast- owing cell also has two chromosomes, each half-replicated, immedi- ely after division.

Coupling between DNA replication and cell division

vision normally follows the completion of each round of chromosome olication. It has also been shown in *E. coli* that completion of a round replication is a necessary prerequisite for division (Clark, 1968; elmstetter & Pierucci, 1968). Thus, if termination of a round of olication is prevented (by specific inhibition of DNA synthesis) then e cell division, which would normally follow 20 min after that termi- tion, is also prevented. Each division event is connected in this way the termination of a specific round of chromosome replication and t to DNA synthesis *per se*. This was shown by inhibiting DNA ıthesis in fast-growing cells where DNA synthesis is continuous (see ove). If a termination has taken place less than 20 min before the ıibition of DNA synthesis, then one division can take place even ough subsequent rounds of replication have been blocked.

It is clear therefore that the processes leading to each specific cell vision become uncoupled from the process of DNA replication at the mpletion of the duplication of a specific chromosome. Alternatively, e termination of each round of chromosome replication produces actly enough of some 'division substance' for one division only.

Evidence has been obtained in this laboratory that the transcription a specific gene (or genes) is required if cell division is to be completed d that this transcription normally takes place at, or immediately after, mination of each chromosome round (Jones & Donachie, in prepara- n). This was demonstrated in the following way.

An asynchronous population of cells of *E. coli* was first synchronised chromosome replication by inhibiting protein synthesis for a period time sufficient to allow completion of all rounds of replication. rther DNA synthesis was then prevented (by thymine deprivation or e addition of nalidixic acid) and protein synthesis allowed to resume. e mass of the population was allowed to double in the absence of NA synthesis. (This period of protein synthesis is also required for bsequent cell division as will be discussed in the following section.) this time all cells have reached or surpassed the required initiation

mass (see previous discussion) and chromosome replication is therefo initiated in every cell when the block to DNA synthesis is removed. protein synthesis is also allowed to continue, then cell division tak place, synchronously, almost immediately after the completion of th round of chromosome replication (Donachie, Hobbs & Masters, 196 Donachie, 1969). However, if further protein synthesis is prevent during this period at any time up to the time of termination, then c division is prevented. Experiments involving the addition and remov of various inhibitors of protein or RNA synthesis for various periods time during this synchronous round of chromosome replication sho clearly that cell division can take place only if there is a short peri (5 min or less) of transcription and protein synthesis subsequent termination of the round of chromosome replication.

It is interesting to note that, under these conditions, there is only very short gap between termination of chromosome replication and c division. It is clear that the normal delay of 20 min is not obligatory

The nature of the 'termination protein' is not known at prese (although preliminary labelling experiments suggest that it may identified as one of the protein components of the cell envelope). O possibility is that it has something to do with a change in state of the c membrane at termination which is required for cell division to completed. For example, this might involve the dissociation of t chromosome from the old replication complex and the subseque conversion of this complex into a septum (Donachie, 1969; Donachie Begg, 1970). It is clear however that this event is required to allow t final assembly of preformed membrane proteins and mucopepti precursors into the septum.

Another interesting question raised by the synthesis of the terminati protein is that of the mechanism of its induction. If its synthesis is tru dependent on the replication of the chromosome terminus, then this m represent a novel control mechanism for gene transcription.

The division clock

As we have seen, cell division normally takes place about 60 min af each doubling in number of initiation mass equivalents (i.e. at $2^n . M_i$ 60 min). The interesting fact is that this time interval appears to constant at different cellular growth rates (with the exception once aga of very slowly growing cells). Since the first two-thirds of this interv runs concurrently with chromosome replication and since termination a necessary event for cell division, it has been suggested that t chromosome replication cycle could provide the clock for divisi

Clark, 1968). This seems unlikely for a number of reasons. For example, is model still leaves unexplained the constancy of the normal interval 0 min) between termination and cell division, which would therefore ave to contain a second 'clock' process which was also independent of owth rate. Also, in the experiments on the role of the termination otein, outlined above, it was shown that division could follow almost mediately after termination if division had previously been prevented inhibition of DNA synthesis. In this case it is evident that most of ese processes which normally take place between termination and vision had taken place *before* termination, i.e. the division clock had en running in the absence of chromosome replication. In addition to ch evidence, certain mutants of *E. coli* exist in which cell division can ntinue after a block to DNA synthesis. In these mutants, since romosome duplication has been prevented, normal sized but DNA-less lls are produced (Inouye, 1969; Hirota, Ryter & Jacob, 1968). Thus e timing of division is normal in these mutants although the chromo-me replication cycle has been prevented. Even more clearly, wild-type *acillus subtilis* are able to continue to divide and give rise to normal zed but anucleate cells when DNA synthesis has been specifically ocked (Donachie, Martin & Begg, 1971).

There is therefore probably a clock for division which is separate from at for DNA replication. The nature of the clock is unknown but periments on the necessity of prior protein synthesis for division ierucci & Helmstetter, 1969) strongly suggest that a constant period of otein synthesis of about 40 min is required before division can take ace. That this is part of a time 'clock' is shown by the fact that this quired period is constant at different growth rates.

This required period of protein synthesis is then followed by an terval of about 20 min during which further protein synthesis (except r the termination protein) is not required. This period therefore esumably represents some process involving the assembly or modifi-tion of preformed proteins into some structure needed for cell division. ne final step in this process would then require the participation of the rmination protein (which, in the normal cell cycle, would have been oduced about 20 min before).

This scheme for the cell cycle in bacteria is outlined in Fig. 4.

The localisation of division

ne division of bacteria is almost always in such a position as to give o daughter cells of equal volume. In the best studied species (*Strepto-ccus faecalis*, *Bacillus subtilis* and *Escherichia coli*), the cells are rods or

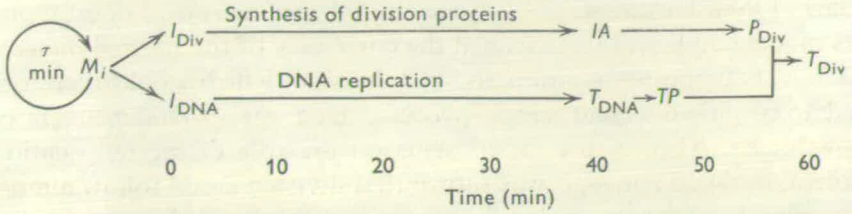


Fig. 4. Model of the cell cycle in *Escherichia coli*. Doubling of the initiation mass (M_i) tak place every mass doubling time (τ min). At each doubling two processes are initiat approximately simultaneously. These are the initiation of DNA replication (I_{DNA}) and t initiation of the sequence of events leading to division (I_{Div}). Termination of chromoson replication (T_{DNA}) at 40 min induces the synthesis of termination protein (*TP*). The fi 40 min of the division sequence involves protein synthesis which is then followed by t initiation of assembly (*IA*). (N.B. Assembly could actually start earlier.) After 15–20 m more the cell has reached a stage (P_{Div}) where interaction between some septu 'primordium' and termination protein leads to cell division (T_{Div}).

Our picture of the timing of the main events in the cycle is therefore of a periodic eve which occurs at intervals equal to the mass doubling time of the cell and at multiples (2 of a constant cell mass (M_i). This event triggers two parallel but separate sequences of ever which take *constant* periods of time to complete, largely independent of the rate of c growth. One of these processes is chromosome replication and the synthesis of the tern nation protein, and requires 40–45 min to complete. The other is a sequence of prote synthesis, followed by another process which may be assembly of some septum precurso This sequence requires nearly 60 min and, at the end of it, there is an interaction betwe the septum precursor(s) and the termination protein to give the final septum and c division. This last event takes only a few minutes.

spheres which divide successively in the same plane (relative to precedi division planes). Other bacteria, like many eukaryotes at early cleava stages, divide successively in different planes, often at right angles to o another. Such species (e.g. *Micrococcus radiodurans*, *Gaffyka*, *Sarcin* etc.) divide and grow to give rise to ordered arrays of cells in either tw or three dimensions. So far, work has concentrated on the simplest ca of cells in which the plane of successive divisions does not vary.

Cell division is a process which involves the invagination of the out layers, or 'envelope', of the bacterial cell. In *E. coli* (and in other gra negative organisms), this envelope is complex in structure but, to a fi approximation, may be considered to consist of three main layers (f review, see Rogers, 1970). These are the inner membrane (lipoprotei outside this is the rigid 'sacculus' (consisting of mucopeptide cross-link polymers) and outside this again the 'outer membrane' (lipoprotein a lipopolysaccharide). This is a highly simplified picture of the c envelope but one which is adequate for our present discussion. T envelopes of gram-positive cells (such as *Streptococcus* and *Bacillus*) a somewhat simpler and may be pictured as an inner cell membra enclosed in a thick 'wall' composed mainly of mucopeptide and teicho

cids. In all cases, however, cell division involves the co-ordinate growth f these layers at a unique site in the cell envelope. The present section is oncerned with the question of how the location of this site is determined.

Two main groups of hypotheses have been considered. The first is at the site is determined by its distance from the cell poles. When the oles are far enough apart, a site for cell division somehow forms etween them. (Note that this does not imply that division will take ace at the same time. The site is only a 'potential division site' until e division clock, discussed above, determines the actual moment of vision.) Such a process would presumably imply the existence of some adient of chemical activity or structural deformation extending out om each pole. A gradient model of this kind would be feasible, no atter how the envelope was synthesised (i.e. by intercalation of new aterial at many sites or by growth in one or a few zones).

The second type of hypothesis is that there are only a few growth sites the cell envelope (in one or more of the layers) and that the potential vision site is located either at such sites or at the consequent junctions etween new and old cell envelope areas.

Various techniques have been employed to determine the mode of owth of the cell envelope, with differing results in different species. ole (1965 for review) and others have used immunofluorescent belling of specific surface antigens to follow the distribution of pre-isting envelope material during cell growth and division. Electron icroscopy has been used, in conjunction with various techniques for arking the pre-existing cell surface, by others (see Ryter, 1968; Higgins Shockman, 1971 for reviews). Radioactive labelling of various velope components (e.g. lipids, proteins and mucopeptide) has been ed extensively. Finally other techniques such as the assay of the stribution of various surface antigens (Autissier, Jaffe & Kepes, 1971) d the measurement of the location of autolytic sites during growth Donachie & Begg, 1970) have been used in some cases.

This work has so far produced a clear picture of envelope growth only r *S. faecalis* (see Higgins & Shockman, 1971). In this nearly spherical ll, growth takes place by addition of new wall material to a central ng which represents the location of the next septum. Autolytic enzymes eave this new material, which then peels apart to form new wall for e two growing halves of the cell. When cell volume has doubled, the dition of new material at the central ring seems to accelerate (or the te of cleavage decreases) so that the ring grows inwards to form a ptum. Autolytic cleavage then splits the septum in two to separate the ughter cells. At the same time, new wall growth begins in each

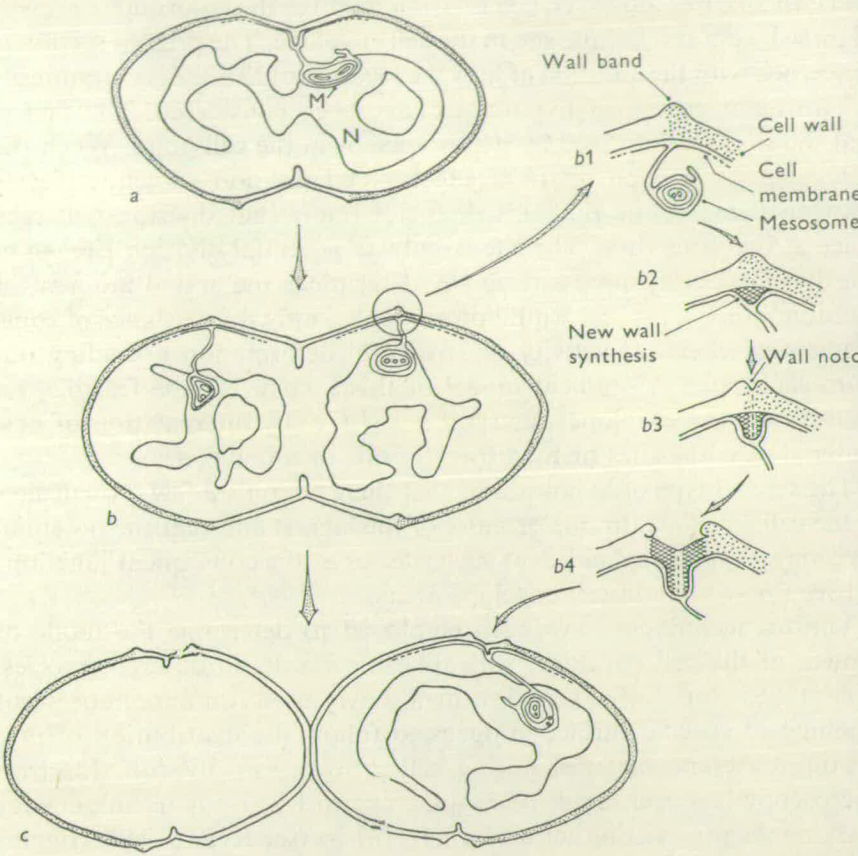


Fig. 5. Growth of the cell envelope during the cell cycle of *Streptococcus faecalis* (taken fr
Higgins & Shockman, 1971). The model proposes that linear wall elongation is a unit
process which results from wall synthetic activity at the leading edges of the nascent cr
wall. The diplococcus in *a* is in the process of growing new wall at its cross wall and seg
gating its nuclear material to the two nascent daughter cocci. In rapidly growing exponen
phase cultures before completion of the central cross wall, new sites of wall elongation
established at the equators of each of the daughter cells at the junction of old, polar w
(stippled) and new equatorial wall beneath a band of wall material that encircles
equator (*b*). Beneath each band, a mesosome is formed while the nucleoids separate and
mesosome at the central site is lost. The mesosome appears to be attached to the plas
membrane by a thin membranous stalk (*b*1). Invagination of the septal membrane appe
to be accompanied by centripetal cross wall penetration (*b*2). A notch is then formed at
base of the nascent cross wall which creates two new wall bands (*b*3). Wall elongation at
base of the cross wall pushes newly made wall outward. At the base of the cross wall,
new wall peels apart into peripheral wall, pushing the wall bands apart (*b*4). When su
cient new wall is made so that the wall bands are pushed to a subequatorial position (e
from *c* to *a* to *b*) a new cross wall cycle is initiated. Meanwhile the initial cross wall cen
petally penetrates into the cell, dividing it into two daughter cocci. At all times the body
the mesosome appears to be associated with the nucleoid. Doubling of the number
mesosomes seems to precede completion of the cross wall by a significant interval. Nucle
shapes and the position of mesosomes are based on projections of reconstructions
serially sectioned cells. Reprinted from *Critical Reviews in Microbiology*, **1**, 1, 1971
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permission.

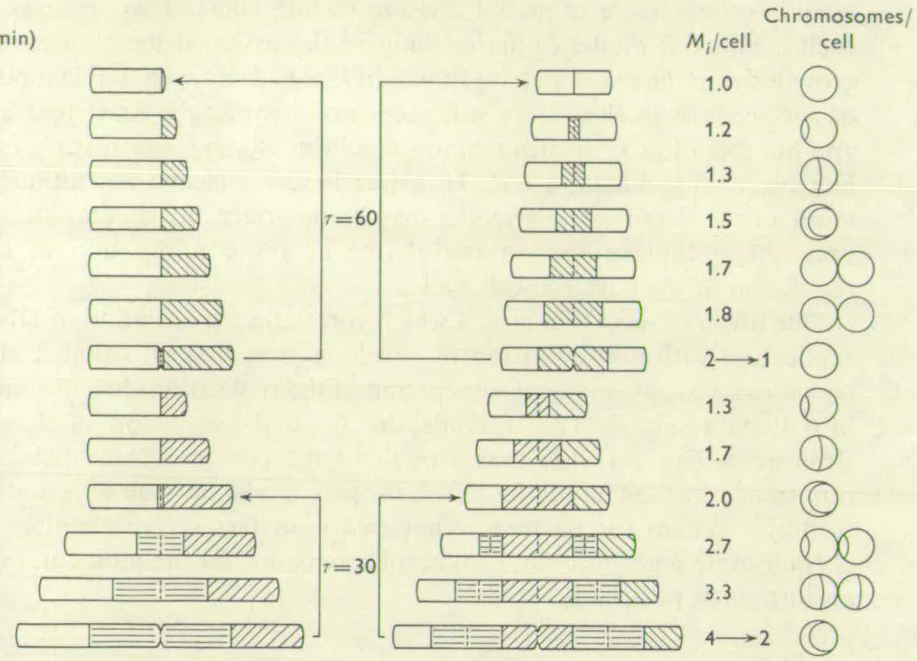
ughter cell at the junction between the wall formed in the cell cycle
ıich has just finished and that which was formed in previous cycles.
ıese new sites are again in the centre of each of the new cells (Fig. 5).
The work on the more extensively studied species, *B. subtilis* and
coli, has unfortunately not yet produced such a clear picture. How-
er in *B. subtilis*, Ryter (1971) has obtained evidence that preformed
gella (in a mutant temperature-sensitive for flagellar synthesis) are
stributed amongst daughter cells in a way which strongly indicates
ıe or two localised regions of envelope growth per cell. In *E. coli*
idence for the existence of a small number of localised regions of
owth has come from studies on the distribution of permease molecules
ocated in the inner membrane) amongst progeny cells (Autissier *et al.*
71). Although there are estimated to be several hundred permease
olecules per cell, these were found to be all distributed into only 50 %
progeny cells after two or three generations (the exact number of
nerations depending on the growth of the cells and hence on cell size).
ıe number of cells containing permease thereafter remained constant.
ther evidence for a localised site of mucopeptide synthesis, located in
e centre of cells, has recently been obtained in pulse-labelling experi-
ents using [^{3}H]DAP (diaminopimelic acid) by autoradiography of the
olated sacculi (Schwarz, Ryter & Hirota, personal communication).
vidence for conserved segregation of large areas of the outer membrane
ıring cell division has been obtained by Leal & Marcovich (1971), who
llowed the segregation of receptor sites for phage T_6. Donachie &
gg (1970) have measured the location of a site of autolytic activity in
e envelope during growth of single cells and concluded, from the
nstancy of the distance between this site and one of the cell poles
ıring cell elongation, that growth of the envelope must necessarily be
king place at a localised site and in a polarised manner (see below).
Such observations suggest that growth of some or all of the envelope
yers takes place at only a few sites in these organisms, as well as in
reptococcus. However, other experiments have produced conflicting
sults. Thus, immunofluorescence labelling of the *E. coli* envelope
eachey & Cole, 1966), radioactive labelling of the mucopeptide (van
ıbergen & Setlow, 1961; Lin, Hirota & Jacob, 1971) and of the
velope lipids or proteins (Lin *et al.* 1971) has shown only a random
spersal of labelled material amongst progeny cells during successive
ll divisions. Such observations have been interpreted in terms of
ffuse intercalation of new envelope components at numerous sites all
er the cell surface. However, none of these experiments excludes the
ssibility that there is extensive turnover of envelope components, if

such components are used preferentially in further envelope synthes
Indeed, the recent experiments of Ryter, Schwarz & Hirota have shov
that localised incorporation of DAP is only demonstrable in sho
pulses. If this newly incorporated material is followed by a chase of u
labelled DAP, label becomes distributed over the whole cell surface
some process which is as yet not understood. This additional observ
tion explains why the earlier experiments on DAP labelling showed
localisation of mucopeptide synthesis. This example clearly shows t
difficulties involved in labelling a highly dynamic structure such as t
cell envelope.

The problem of the exact mode of growth of the cell surface is ther
fore not yet solved but the balance of the evidence is, at the moment,
favour of the existence of a few localised sites of envelope growth in t
envelope of these bacteria so far studied. This does not mean to say th
there are not also other processes going on which involve the tran
location of some envelope components from one location to anoth
However such a form of growth does imply the existence of junctio
between new and older materials at precise locations in the cell surfa
Such junctions could provide the spatial information required for t
localisation of cell division. (See Higgins & Shockman, 1971 for mo
extensive discussion.)

Bacteria may contain more than a single site of potential divisio
depending on the size of the cell. This has been shown in *E. coli* in whi
division has been blocked specifically (by low concentrations of pe
cillin) while cell growth was allowed to continue. The number a
location of the potential division sites was then determined, for cells
different lengths, by releasing the inhibition and measuring the dividi
cells. In this way it was found that the number of potential division si
increases at each doubling of a basic cell volume. This basic volume w
found to be equivalent to that of a cell of mass equal to the initiati
mass (M_i) for DNA synthesis (Donachie & Begg, 1970 and unpublishe
Therefore at the same cell sizes ($2^n . M_i$) at which new rounds of chrom
some replication and the division clock are initiated, new sites for c
division are formed.

Such sets of observations have been combined into a 'unit cell' mo
for bacterial growth (Donachie & Begg, 1970) in which it is assum
that a periodic event in the cell envelope (occurring at $2^n . M_i$) results
a doubling of each pre-existing envelope growth site, giving rise to t
new membrane replication complexes at which new rounds of chrom
some replication can be initiated and also resulting in the conversion
the pre-existing growth site into a site of potential cell division (whi



g. 6. Growth of the cell envelope in *Escherichia coli* according to two variants of the unit ll model.

At 0 min a unit cell (mass/cell = M_i) is inoculated into a medium where the mass ubling time (τ) is 60 min. After division at 60 min, one of the two daughter cells is oculated into a richer medium where τ = 30 min. The first division in this new medium kes place 60 min after this, by which time the new daughter cells are each 2 unit cell uivalents in mass. (For simplicity, cell mass is drawn as proportional to cell length. In fact ll diameter also increases on transfer to richer medium so that the relative proportions of lls with different growth rates are somewhat different from those shown. The relative cell asses are however as shown.)

The steady-state cell cycles in the two media are shown by large arrows.

Vertical solid lines across the cells show the edges of the growing zones in the cell envelope. ıe number of these zones doubles whenever the number of unit cell equivalents doubles. The rate of increase in cell volume (length in this figure) is assumed to be proportional to e number of growth zones and also to τ. The rate per zone therefore depends on the owth medium but the rate of volume increase always doubles at each doubling in unit cell uivalents.

Cell division takes place 60 min after each doubling in number of unit cell equivalents, at osition which corresponds to the position of the edge of the growing zone 60 min earlier. is position (the future site of cell division) is shown throughout as an interrupted vertical e.

The location of each successive cycle of growth is shown by a different direction of cross-tching.

The left-hand sequence represents cell growth according to a model in which a unit cell s a single growth zone located at one pole (Donachie & Begg, 1970). The other sequence resents the possibility that each unit cell has a central growth zone (Autissier, Jaffe & epes, 1972). Both models provide a possible way in which the location of the site of cell vision could be determined by localised regions of growth of the cell envelope.

The number of chromosomes per cell, together with their state of replication, is also own, on the assumption that initiation takes place at every doubling in number of unit l equivalents and that each round of replication takes 40 min to complete.

would become a site of actual division 60 min later). Two versions
such a unit cell model (differing only in the assumed location of t
growth site in the envelope) are shown in Fig. 6. The two possible mod
of surface growth shown are still necessary, because the exact locati
and number of growth sites remains a subject of some controversy (s
Higgins & Shockman, 1971). However it seems clear that, althou
many of the details of this model may be incorrect, the general idea
unit cell multiplication is a useful one in any consideration of t
regulation of the bacterial cell cycle.

One further aspect of a model which correlates the initiation of DN
replication with multiplication of envelope growth sites is that it al
provides a possible mode of segregation of the replicating chromosom
into daughter cells. This extends the original suggestion of Jaco
Brenner & Cuzin (1963) that growth of the cell membrane betwe
points of attachment of the chromosomes could provide a primiti
'mitotic' system for bacteria. That this is in fact the mechanism
chromosome segregation in prokaryotes remains, for the moment, on
an intriguing possibility.

ENZYME SYNTHESIS DURING THE CELL CYCLE

The bacterial cell cycle is often described only in terms of DNA rep
cation and cell division, but it is now clear that the overall compositi
of the cell also changes in respect of many enzymes not obvious
directly involved in either of these processes.

One possible source of this variation is an obvious consequence of t
sequential replication of the genome, in that the potential to synthes
any enzyme ought to double when its structural gene is replicated. T
has been shown to be the case by experiments in which the induced
derepressed rates of synthesis of several enzymes have been measured
various points in the DNA replication cycle. The times at which th
rates double have been shown to correspond to the times of replicati
of the corresponding structural genes (Masters & Pardee, 196
Donachie & Masters, 1966; Pato & Glaser, 1968; Helmstetter, 196
If the numbers of genes were the only factors determining the rate
enzyme synthesis then one would expect that every enzyme would
synthesised continuously throughout the cell cycle. In this simplest ca
the rate of synthesis should be linear with a doubling in rate at the ti
of gene duplication. Although this pattern has been seen in several cas
the synthesis of many other enzymes has been shown to follow oth
patterns.

Two basically different systems have been investigated: synchronous getative cultures of *E. coli*, *B. subtilis* or *Rhodopseudomonas spheroides*, d synchronous spore outgrowth in the genus *Bacillus*. Three methods e commonly used to prepare synchronous vegetative cultures: the lution of stationary phase cultures into fresh medium (Yanagita & aneko, 1961; Masters, Kuempel & Pardee, 1964; Cutler & Evans, 66), the membrane elution technique of Helmstetter & Cummings 963, 1964), and size selection using sucrose gradient centrifugation Iitchison & Vincent, 1965). The latter two methods have the advantage minimising metabolic disturbances which can seriously affect the nthesis of many enzymes, while the starvation and dilution method can oduce an almost perfect synchrony that will persist for many genera- ns. A list of the enzymes studied, together with their mode of nthesis, can be found in a recent book by Mitchison (1971, p. 176).

As has been mentioned above, the synthesis of many enzymes in nchronous cultures does not follow the simple pattern of constant nthesis with a doubling in rate at the time of gene duplication. These zymes are synthesised in a brief burst during the cell cycle, so that the nount of enzyme in the cell is constant for part of the cycle, doubles ruptly, and then is constant for the rest of the cycle. In a synchronous pulation of cells the total amount of such an enzyme rises in a series steps, with one step in each cell cycle. In all cases which have been vestigated it has been found that such periodic enzyme synthesis takes ace under conditions where a regulatory feed-back system is operating. ch conditions, which have been called 'autogenous' because the rate enzyme synthesis is then determined in part by the rate at which the zyme produces its own end-product repressor (Masters & Pardee, 65), are the usual ones under which most biosynthetic enzymes are oduced in growing cells.

The fact that such periodic synthesis is not seen under conditions of nstant repression or derepression has given rise to the idea that this riodic synthesis is the consequence of oscillations in the normal feed- ck regulatory system (Masters, Kuempel & Pardee, 1964; Kuempel, asters & Pardee, 1965; Masters & Donachie, 1966). Indeed, computer udies have shown that such oscillations are an intrinsic property of mple feed-back systems, if suitable constants are assumed (Pardee, 66; Goodwin, 1966). Such a model for periodic enzyme synthesis has en supported by the observations that addition of inducer can stimu- e synthesis of such enzymes and addition of end product can abolish nthesis at all times in the cell cycle (see Donachie & Masters, 1969 for view). Also, the timing of the periods of synthesis in the cell cycle can

be displaced for specific enzymes by a single pulse of repressor (Maste & Donachie, 1966).

The observed periodicity of autogenous enzyme synthesis is the sar as that of the cell cycle, but this is not a necessary attribute of oscillati feed-back systems. It is therefore necessary to explain this periodicity some other mechanism. Goodwin (1966) has proposed that the regul periodic duplication of individual genes could provide an entrainme mechanism which would make the periodicity of enzyme synthesis equ to that of gene duplication.

This model would also predict that, since the replication of t bacterial genome is sequential, the order of synthesis of various bi synthetic enzymes under autogenous conditions could be the same as t order of replication of the corresponding structural genes. Just such correspondence was reported by Masters & Pardee (1965) for thr enzymes and their genes in *B. subtilis*. A similar correlation has recen been reported for five enzymes in germinating spores of *B. subti* (Kennet & Sueoka, 1971). However it seems clear that, in this lat example at least, the order of enzyme synthesis cannot be determined entrainment mechanisms of the kind envisaged by Goodwin, since ge duplication in germinating spores does not begin until *after* the fi sequence of enzyme synthesis has been completed. The situation germinating spores is different from that in vegetative cells in that t synthesis of at least some enzymes cannot be induced at all times b only at times corresponding to their periods of 'spontaneous' synthe (Steinberg & Halvorson, 1968*a*, *b*). The possibility therefore exists th in this situation, the genome becomes available for transcription in ordered fashion so that individual genes are transcribed in sequen There are, however, other less dramatic explanations possible for su variations in inducibility of enzymes, including fluctuations in catabol levels during spore germination. Such classical explanations must excluded before it is necessary to hypothesise that some novel mechani of gene transcription is taking place during spore outgrowth.

Outgrowth is well characterised in terms of morphological chang RNA, DNA and protein synthesis (see Hansen, Spiegelman & H vorson, 1970; Keynan, this Symposium p. 97). The dormant spo contains little or no functional mRNA though it does contain RN polymerase (Sakakibara, Saito & Ikeda, 1965). Overall protein synthe is continuous during outgrowth but only a very few kinds of prot synthesised in the early stages (Kobayashi *et al.* 1965) and the prote being synthesised change with time (Torriani, Garride & Silberste 1969). It has also been shown that the species of mRNA being sy

esised change with time (Hansen, Spiegelman & Halvorson, 1970). einberg & Halvorson (1968*a*, *b*) studied the timing of synthesis of rtain enzymes during outgrowth. They found not only that synthesis these is normally restricted to a certain period during outgrowth but, at in the case of the two inducible enzymes studied, they were in- ucible only during this restricted period.

It is known that the RNA polymerase of *B. subtilis* is altered in the β bunit during the process of sporulation and differs in template ecificity from the polymerase of the vegetative cell (Losick & Sonen- ein, 1969; Sonenshein, Losick & Shorenstein, 1970). Presumably the NA polymerase found in the spore, during early outgrowth at least, of this kind but it is easily imaginable that a third form of RNA lymerase might be responsible for the ordered transcription found ring outgrowth (Kennet & Sueoka, 1971). However such a mechanism nnot explain the ordered enzyme synthesis found during the vegetative cle in *B. subtilis* (Masters & Pardee, 1965), since, as we have said, under ese conditions it has been shown that any enzyme can be induced or pressed at any time during the cell cycle. Therefore if there is a quential reading mechanism responsible for the order of enzyme nthesis in the vegetative cell cycle it must coexist with normal feed- ck systems controlling transcription of these genes. It is difficult to visage the operation of such a dual system and even more difficult to visage the necessity of such systems for the temporal control of the ll cycle. For both of these reasons it is best to admit that the regulation enzyme synthesis in the cell cycle is not fully understood.

It is clear that while a great deal of work remains to be done in scribing variations in enzyme levels during the cell cycle, those zymes which have been studied up till now have little or no direct role either chromosome replication or cell division. Unfortunately there e as yet no suitable methods of assaying enzymes directly involved DNA replication or cell division.

At least some autolytic enzymes have been shown to become active at particular time in the cycle and to act only at the position of the pre- mptive site of septum synthesis (Schwarz, Asmus & Frank, 1969; onachie & Begg, 1970; Hoffmann, Messer & Schwarz, in preparation). owever the role of these enzymes in cell division is not definitely tablished.

There is at present therefore no information about the spatial or mporal control of the enzymes directly concerned with the major ents of the cell cycle.

CAULOBACTER

The bacterium *Caulobacter* provides a very convenient system by whi the control of cellular differentiation can be investigated, though i potential as a source of information is only beginning to be realised. T cell cycle is unique in that the vegetative cycle consists of two cell typ a motile 'swarmer' cell and a non-motile 'stalked' cell. Synchrono populations are readily obtained by selecting newly formed motile ce (Newton, 1972) and the system is susceptible to analysis by the esta lished techniques of bacterial genetics.

The life cycle of *Caulobacter crescentus* has been described elsewhe (Poindexter, 1964; Shapiro, Agabian-Keshishian & Bendis, 1971) a will be only briefly summarised here (see Fig. 7). Only the stalked c can divide, giving rise to two very different daughter cells; another stalk cell and a motile 'swarmer' cell. The swarmer cell has a single pol flagellum and a holdfast (a 'sticky' region) near the base of the flagellu After a brief period of motility the swarmer cell loses its flagellum a forms a stalk at the site of the holdfast. The stalk is a continuation of t lipopolysaccharide and mucopeptide layers of the cell wall and associated with a complex intracytoplasmic membrane structure at base (Schmidt & Stanier, 1966). This stalked cell can now begin tho processes of the cell cycle which are common to the two sister cel including DNA replication, the synthesis of many pili, and the synthe of the flagellum, holdfast and basal membrane structure at the 'u differentiated' pole. The sister stalked cell begins these proces immediately; thus the time of division of the two sister cells differs the time required for the morphogenesis of the swarmer cell to the stalk form. Hence, unlike the bacteria most usually studied, the vegetati cycle in *Caulobacter* shows a number of morphological differentiati events occurring in a fixed temporal sequence.

Newton (1972) has recently investigated the role of transcription the control of this cycle using the antibiotic rifampicin, which specifica inhibits the initiation of transcription (Wehrli, Nüesch, Knüsel Staehelin, 1968). His results are summarised in Fig. 7. Each step development could be inhibited by the addition of rifampicin up to critical point in the cell cycle. Addition after this time had no effect. F example, division and stalk formation could be inhibited up to 10 m before the event, loss of motility up to 40 min before the event, a DNA synthesis up to the normal time of initiation [similar observatio on a requirement for RNA synthesis for the initiation of DNA synthe have recently been made for the bacteriophage M_{13} by Brutlag, Shekm

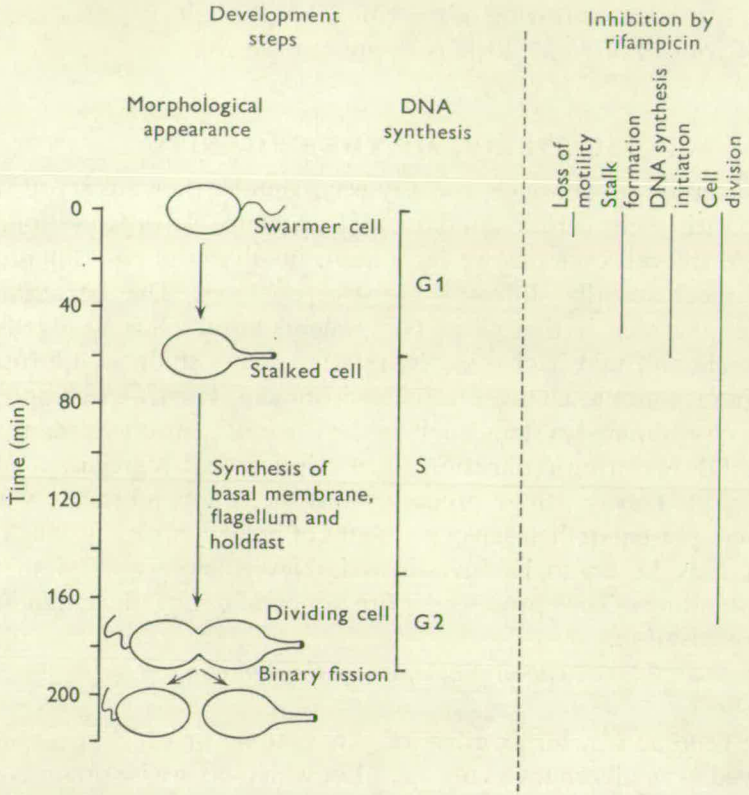


Fig. 7. The cell cycle of *Caulobacter crescentus* (taken from Newton, 1972).

Kornberg (1971) and for *E. coli* by Lark (1972)]. It is interesting to te that while DNA synthesis begins immediately in the stalked cell the armer shows a considerable lag. This differential control of DNA nthesis in the two progeny cells would appear to rule out a model for control of initiation in *Caulobacter* which involved the dilution of a oplasmic repressor during cell growth (as proposed for *E. coli* by tchard, Barth & Collins, 1969).

These studies show that development in *Caulobacter* is controlled, at st in part, by differential gene expression at the level of transcription. owever the wide variation in timing between the transcriptional event d the execution of the step suggests that other levels of control must o operate.

The potentially most important feature of *Caulobacter* in studies of bacterial cell cycle is that, in contrast to other bacteria, the cycle ludes a number of readily visible morphogenetic events with a clearly ined spatial location within the cell. Thus *Caulobacter* provides a good

system for the investigation of the control of spatially oriented events, type of control of which little is at present known.

MYSTERIES OF THE ORGANISM

The bacterial cell, although in many ways simpler than eukaryotic cel shares with them certain fundamental problems of organisation. F example the cell cycle, as we have seen, involves the co-ordination many biochemically different growth processes. The bacterial c behaves as a unit in that all its components are duplicated together each cycle and that it changes its relative composition in appropria ways in response to changes in its environment. Particular compone of this co-ordinated system, such as the three dimensional organisati of the DNA during replication, transcription and segregation or t interactions between these processes and the cell membrane (as in c division), present in themselves problems of great complexity which ha as yet only begun to be investigated. Nevertheless we feel that t understanding of such processes is fundamental to any understanding cell organisation.

The organisation of the genome

Gene order

The genome is a linear array of genes, some of which are spatia arranged in small groups as operons, but which otherwise presents eve indication of being randomly ordered. Thus genes affecting simi processes may be scattered around the whole map. Nevertheless, as have already discussed, this spatial order may be reflected in the tempo order of synthesis of several enzymes during the cell cycle. Such ordered sequence of change in levels of different enzymes may well pl a role in the ordering of other necessary events in the cell cycle. T possibility therefore exists that the developmental sequence of the c cycle may be determined in part by the linear sequences of genes on t chromosome. It should be noted that a fixed sequence of synthesis different enzymes is also a characteristic property of the eukaryo cycle (see Mitchison, 1971), as well as of developmental sequences general.

Another way in which the location of certain genes on the chron some may be important in the regulation of cell function is as a co sequence of the change in relative proportions of different genes different growth rates. At fast growth rates there are more replicati forks per chromosome than at low growth rates (see above and Fig. and, consequently, relatively more copies of genes close to the ori

an of those close to the terminus. This change in relative proportions most genes will presumably be compensated to some extent by the eration of the normal feed-back systems of end-product inhibition d repression. However, if there are genes in which no such regulatory stem operated, this change in relative proportions will presumably ult in a change in the relative proportions of their gene products in e cell (Sueoka *et al.* 1970; Van Dijk-Salkinoja & Planta, 1971; Hughes, 71). Thus the genes coding for ribosomal proteins and ribosomal RNA e close to the origin in both *B. subtilis* (Oishi & Sueoka, 1965; Smith *al.* 1969) and *E. coli* (see Taylor, 1970). If, as seems likely, these genes e 'constitutive' under all cell growth conditions, then the relative portion of ribosomes should increase with increasing growth rate. st such an increase is observed (Maaløe & Kjeldgaard, 1966). terestingly, not only is an increased concentration of ribosomes within e cell a necessary consequence of increased growth rate (because of this ange in gene proportions) but it is in turn also itself a prerequisite for ch an increase in growth rate. This is because the rate of polypeptide ngation per ribosome is approximately constant at different growth es and therefore any increase in rate of protein synthesis requires an reased number of ribosomes per cell (Lacroute & Stent, 1968). This mple shows clearly the mutual interdependence of different processes he cell. To decide whether this proposed mechanism for the regulation ribosome synthesis is correct will now require the experimental trans-ation of ribosomal genes relative to the chromosome origin.

plication

The process of DNA replication has always represented a conceptual blem because of the obvious mechanical difficulties involved in licating a double helix of such length (1100–1400 μm). The problem s been discussed in some detail by Watson & Crick (1953), Delbrück & nt (1957) and Cairns & Davern (1968). Two models that were posed many years ago, breakage-and-reunion (Delbrück & Stent, 57) and various forms of active unwinding of the helix (Levinthal & ane, 1956; Cairns, 1963; Maaløe & Kjeldgaard, 1966) have still not en tested.

Sequential replication of DNA requires that daughter strands of posite polarity be synthesised concurrently; i.e. in the 5′-to-3′ and in 3′-to-5′ direction. The DNA polymerases characterised to date, from sources, will proceed only in the 5′-to-3′ direction in *in vitro* systems. ere have been many models suggested to explain this discrepancy, ugh the possibility remains very strong that we have not yet isolated

and characterised correctly the enzyme(s) actually responsible f normal DNA replication. Sugino & Okazaki (1972) have succeeded showing that T_4 phage DNA synthesis proceeds only in the 5′-to direction.

Most models for DNA synthesis assume that synthesis can in fact o proceed in the 5′-to-3′ direction and suggest some form of discontinuo synthesis of one or both daughter strands (for a recent review see Gro 1972). Those models all suggest that one implication of a single directi of synthesis is an asymmetry in the mode of replication of the t daughter strands, and there are a few experimental observations th suggest that a basic asymmetry exists in some phage systems (Inman Schnös, 1971). The evidence in bacterial systems is not so clear bu consensus appears to be emerging that one of the two daughter strar is synthesised continuously, the other discontinuously (see Gross, 197

Physical organisation of the DNA in the cell

The bacterial genome consisting of a single DNA molecule nea 1000 times the length of the cell itself is condensed into a 'nuclear' regi that is of the order of 0.5 μm in diameter. Maaløe & Kjeldgaard (19 have pointed out that in such a structure the average distance betwe adjacent daughter strands of DNA must be no more than 30 Å. Wh most electron micrographs show an apparently disordered arrangem some show an approximately ordered arrangement. If it is difficult imagine the replication of an extended DNA molecule in solution i much more so in a dense and presumably ordered structure. The rate movement of a replication fork is about 14 μm/min (40000 base pa min), and throughout this process the nuclear material retains condensed structure. It is obvious that this must involve contin breaking and remaking of bonds of some sort between all parts of structure. In speculating about the nature of the condensed state i interesting to note that when DNA replication is specifically inhibi the dense packing is quickly lost and loosely packed DNA fibrils formed throughout the cell (Donachie, Martin & Begg, 1971; published observations). Fong (1967) suggested that the comp structure could be the result of supercoiling induced by the rewinding the duplex during replication, which would be consistent with observation that such condensed structures disappear when replicati stops. However, Stonington & Pettijohn (1971) succeeded in isolat the genome of *E. coli* as a folded DNA–RNA–protein complex. Th found that the structure was dependent on RNA for the maintenance its integrity, in that ribonuclease treatment caused rapid unfolding. T

ggests that supercoiling is not the sole reason for the condensed ructure of bacterial DNA in the cell.

The extent of our ignorance about DNA replication, even in bacteria, emphasised when it is realised that not only is the DNA in constant otion within the nucleoid relative to the replication fork, but that nultaneously the two growing daughter strands are being segregated gain by an unknown mechanism) in a process that eventually results in e formation of two separate nuclei.

Maaløe & Kjeldgaard (1966) have also pointed out that if the spacing tween DNA helices is regular then it is not possible for RNA polymer- e or ribosomes to enter the nuclear region. This would suggest that ly genes which were at the surface of the nuclear region could be nscribed. Nevertheless many experiments have shown that any gene n be transcribed at all times in the cell cycle if it is specifically induced derepressed. Therefore every gene must be available for transcription least in every few minutes throughout the cell cycle. Whether this lects yet another dynamic process of the condensed DNA or whether indicates that, for example, all operators are exposed at the surface, unknown. In this respect it is interesting to note that the diameter of e bacterial nucleus is of the order of the length of the average gene. erefore it is possible for every gene to have at least one end at the rface of the nuclear region. The process of transcription could then volve the attachment of the polymerase leading to the local unfolding the genome.

This brings one to the problem of recognition of specific regions of the NA by proteins involved in DNA replication, transcription, modifi- ion, and restriction. In principle such recognition could be for ecific linear sequences of bases, but another attractive possibility is t recognition sequences may cause specific alterations in the second- structure of the DNA. Brom (1971) has shown that AT-rich regions y have an altered helical structure and it is known that large AT-rich ions do exist in *E. coli* and *B. subtilis* (Yamagishi & Takahashi, 1971). e binding sites for many proteins such as micrococcal nuclease, *lac* repressor, and RNA polymerase are AT-rich and this altered ondary structure may play a role in recognition. It is also possible t localised base pairing within a single strand might play a e.

To truly appreciate the possible roles of the secondary or tertiary ucture of DNA (or RNA) in the cell cycle we must turn to recent rk on the small RNA phages such as Qβ and f2. In these phages the NA serves as a template for both transcription and translation. It also

contains recognition and protein binding regions and extensive regio of secondary structure that contribute to the regulation of transcriptio Only three proteins are coded for by the phage genome (reviewed b Kozak & Nathans, 1972). The present understanding of the role playe by secondary structure in these phages depends on the extensi nucleotide sequence data that are now available. The translational contr depends on the maintenance of a secondary structure that preven ribosomes from reaching the appropriate binding sites, and at least tw of the three proteins specified by the phage genome interact with t genome to alter this secondary structure as the developmental cyc progresses. The process of replication of the RNA also plays a ro Although this extreme genetic economy in regulation is probably n necessary to the bacterial cell it seems possible that regulatory mecha isms involving the secondary or tertiary structure of DNA and RNA m play a role in the regulation of transcription and translation in pr karyotes. Kennell (1968) has shown that a high proportion of the *E. c* chromosome is transcribed only very rarely or not at all. Genes tra scribed at very low frequencies could be dependent on disruption secondary structure, such as that occurring during replication, for th transcription.

Transcription and translation also present mechanical proble related to those presented by DNA replication. The electron micr graphs by Miller and his colleagues (1970) show that RNA polymera molecules follow one another closely on active operons and that rib somes attach to and begin to translate the growing mRNA cha immediately. It is very unlikely that this large complex of RNA po merase, nascent mRNA, ribosomes, and nascent protein molecules co be rotating around the DNA molecule during transcription, so we a once again faced with the problem of either spinning the entire DN molecule during transcription (in both directions depending on wh strand is transcribed for a given gene) or introducing single strand breaks in the DNA molecule.

The organisation of the cell envelope

One of the earliest ideas about the organisation of cells (before t discovery of genes) was that it was in large part determined by the c wall (Schleiden & Schwann, 1838). This idea fell out of favour with t discovery of the importance of chromosomal genes coding for vari elements of the cell but there remains evidence that physical struct serves to some extent as a template for further growth and thus m determine the pattern of that growth (Tartar, 1961; Sonneborn, 196

The shape of the bacterial cell, whether a rod or a sphere, is determined by the rigid mucopeptide layer of the cell envelope. This is clear cause cells in which the mucopeptide has been removed take up a herical shape in liquid while the isolated mucopeptide retains the ape of the intact cell (Schwarz, Asmus & Frank, 1969). However it is ear that the mucopeptide itself does not determine the shape of the ogeny cells since partial or complete removal of this layer does not ange the morphology of progeny cells derived from the altered cell andman, Ryter & Fréhel, 1968). Also, point mutations that cause erations in cell morphology are known (Adler, quoted in Taylor, 70; Alstyne & Simon, 1971), suggesting some form of direct genetic ntrol of cell shape. There is as yet no evidence that physical structures bacteria can serve as a template for subsequent generations. However e structure of the cell surface must be extremely important not only in termining cell shape but also in determining the site of cell division d presumably the site of DNA replication and segregation. The achment of DNA to the cell membrane in bacteria is now well docuented but the original suggestion of Jacob, Brenner & Cuzin (1963) at the DNA is attached to a precise point on the surface whose cation is important for the proper segregation of DNA daughter ands remains at this time no more than an intriguing possibility. esent methods of electron microscopy do not seem to be adequate to ow any precise attachment point.

The one event which is clearly localised on the cell envelope and thus presents a local discontinuity in the structure is septum formation. e way in which the localisation of this site might be determined has eady been discussed but it is clear that we do not know whether the e arises *in situ* in a previously homogeneous envelope or whether the continuity that will give rise to the site of cell division is present oughout the cell cycle (as was suggested, for example, by the work of nachie & Begg, 1970, and Schwarz *et al.* personal communication). the latter case the possibility exists that these discontinuities arise one m another, in a process akin to the duplication of an organelle (for mple, the centriole) in eukaryotic cells, during cell growth. The way which this localised site appears should be an object for fruitful ther study, representing as it does a simple example of morphonesis in the cell cycle.

The events that occur at the site at the time of cell division are complex d apparently involve the synthesis of structures which are basically ntical to the rest of the cell wall but oriented differently with respect he cell axis. It is not known whether they also involve the synthesis of

novel elements, such as contractible fibrils, as they do in eukaryot There is evidence for some structural differences in the cell wall a septum. Fan *et al.* (1972*a*, *b*) have shown that the cell ends are mo resistant to autolytic degradation than the sides, but it is not at prese possible to analyse local variations in structure of so complex an aggr gate of macromolecules in any precise way.

Jacob *et al.* (1963), as mentioned above, suggested that the growth the cell surface in localised zones could serve as a segregation mechanis for sister DNA molecules attached at opposite ends of the growing zo We have seen that the required kind of zonal growth does take place bacteria but that the localised attachment of DNA has not been demo strated. If such attachment occurs it seems unlikely that it can be permanent one since Ryter & Jacob (1966) have shown that particul labelled DNA strands segregate at random at each division. (Howev Eberle & Lark (1966) have presented evidence that is consistent with more permanent attachment.) The recent proof that replication *E. coli* is bidirectional (Masters & Broda, 1971; Bird, Louarn, Mart scelli & Caro, 1972; Hohlfeld & Vielmetter, personal commu cation) makes the model of Jacob *et al.* more attractive. The earl assumption that the origin and terminus of replication were adjace meant that segregation could not begin as soon as DNA replication w initiated, as now appears to be possible.

'*Clocks*'

The duration of the cell cycle is determined in part by the genome a in part by the environment. In bacteria the length of the cycle can very easily controlled by changing the composition of the grow medium and this has been, to a large extent, responsible for the d covery of the various regulatory mechanisms controlling the cell cy which we have outlined in this article. Analogous control systems m be discovered in eukaryotic cells when the length of the cell cycle con similarly under experimental control. Meanwhile the work with bacte has revealed several novel aspects of the cell cycle, including the existe of several clock-like systems which are responsible for the timing various events.

The most obvious of these systems is the process of DNA replicati which, as we have seen, plays a central role in the cell cycle. Unde large number of environmental conditions (at constant temperature) which the growth rate of the cell can vary between about 1 and doublings per hour, each pair of replication forks takes a constant ti to travel from the origin to the terminus of the chromosome (see Fig.

e constancy of the rate of addition of nucleotides to the growing
NA chains is presumably a consequence of the existence, at each
lication fork, of a single replication complex or small number of
zyme molecules which are saturated with substrate over the range of
strate concentrations found in cells at these growth rates. The
neral explanation for the constancy of the rate of DNA replication is
refore probably a trivial one. However the fact that the sequential
plication of the genome is so prolonged, relative to the cell cycle,
plies that the proportions of different genes change both during the
cycle and in cells growing at different rates. We have asked the
estion whether there has been, as a consequence, evolutionary
ection of gene order so that appropriate ratios of different genes will
ur both at different stages in the cell cycle and under different
vironmental conditions. There is presumptive evidence to believe that
latter may be true for the genes concerned with ribosome synthesis
experimental evidence is still lacking to decide whether the order of
es is of any major importance to the cell. The other timed event in
ich the duplication of the genome plays a role is in the induction of
synthesis of a specific protein or proteins which appears to take place
en each pair of replication forks reaches the chromosome terminus.
wever this protein does not seem to be required until much later in
cycle and therefore it is not clear that the exact duration of rounds of
NA replication is important in the timing of division.

The possible reasons why each DNA round takes a constant period of
e in the cell cycle are obvious but it is not obvious that this process is
d as a clock to determine the timing of other events in the cell cycle.
e second clock-like process which we have discussed, the so-called
vision clock' (Fig. 4), is much more mysterious in its molecular basis
its consequences in the cell cycle are much more obvious. The
lecular processes involved are still largely unknown but they include
xed period of protein synthesis and another fixed period which
ght, for example, involve the assembly of a structure from preformed
nents and, in total, these processes require about one hour to
nplete. The peculiarities of these preparations for division are that
y take approximately the same time at different growth rates and
, in fast growing cells with generation times of less than one hour,
h division preparations must have run for one hour before each
sion. This implies that several such processes, each at different
ges, must be running in parallel within the same cell at any instant.
s, in turn, strongly suggests the spatial separation within the cell of
preparations for individual divisions. The simplest model is one in

which assembly of precursors for each septum takes place at a s which represents the future location of that septum. The growth of ea septum would therefore begin one hour before its final completion. (C difficulty in the way of this model is that no sign of such a septu precursor is usually seen in conventional electron micrographs of th sections. However this may well be an artefact of the fixation procedu Steed & Murray, 1966.) If the clock process is indeed the addition newly synthesised (and perhaps unstable) elements to a division p mordium, then the same formal explanation for the constant time fr initiation to completion of the septum can be given as in the case DNA replication. Addition of precursors could take place at a fi number of sites, or be catalysed by a fixed number of enzyme molecul arranged in a ring around the cell circumference (see Fig. 6).

Both DNA replication and the sequence of events leading to divis are initiated at about the same time in the cell cycle (Fig. 6) and possibility therefore exists that they are in fact triggered by the sa event. In the unit cell model, we have assumed that this event is appearance of new surface sites which serve both as attachment sites new rounds of DNA replication and as the eventual sites of cell divis after these rounds have been completed. The problem then arises as how these sites themselves arise. All one knows, at the moment, is t the initiation events take place at every doubling of a unit mass (M which suggests that the process leading to the formation of new s takes place at a rate proportional to the rate of overall cell grov (Fig. 6). This process is therefore not a clock in the same sense as th just discussed, which both proceed at constant rates at different growth rates. Instead of taking place at regular intervals of time, master-process takes place at regular increments of mass. It is a 'm clock', not a 'time clock'. This system is also a replicating system in sense that two new rounds of DNA replication and two new divis sites are generated for each pre-existing site at each doubling of unit mass. The possible molecular basis for such a multiplicative proc of what are presumably structures in the cell envelope remains co pletely unknown. That just such a duplication of surface rings can oc is however clearly seen in the cell cycle of *S. faecalis* (Fig. 5). It would extremely interesting to know whether new DNA rounds are initia in this organism at the same time as the first appearance of the pai new surface rings. Unfortunately there are as yet no studies of this k in this organism.

One other event that takes place at the same time as the initiatio DNA replication is a doubling in the rate of cell growth (Ward

laser, 1971). This supports the original suggestion (Donachie & Begg, 970) that the newly formed surface rings are also sites at which all net owth of the cell surface takes place. The 'clock' for the initiation of vents in the cell cycle is therefore probably also partly responsible for e regulation of overall growth rate.

This discussion suggests that a great deal of the temporal organisation the bacterial cell cycle is the consequence of the spatial differentiation its surface layers. The simple view of the bacterial cell as a membrane closing a number of independently operating biochemical systems, ch specified by the genome, has therefore been replaced by a more mplex picture in which the organisation and localisation of certain mplex enzyme systems within the cell envelope is of key importance in e regulation and integration of the major events in the cell cycle. Every mponent in the cell must be specified by the genome but the spatial rangement of at least some of these components may prove not to determined by the genome but rather by the pre-existing arrangement the same components in parent cells. It will not however be known nether this is true until it is possible to attempt to reconstitute a viable ll from its constituent molecules. If such surface organisation is in this nse 'inherited' in bacteria it is clear that the organisation must be imarily in the membrane layers rather than in the rigid mucopeptide yer, since most of the mucopeptide can be removed without impairing e ability of the resultant protoplast eventually to regain its normal ape on reconstituting the missing layer.

CONCLUSIONS

is article has emphasised the vegetative cell cycle in one or two cterial species but other aspects of differentiation in prokaryotes have en ignored. We have therefore not mentioned the more complex velopmental systems of the Myxobacteria and the *Streptomyces* or process of sporulation in *Bacillus* which are dealt with elsewhere in s Symposium.

We have attempted to survey briefly the main features of the bacterial l cycle, not only to describe those aspects which are well understood t also to emphasise those processes which are very little understood. e have not hesitated to speculate and to construct models of the main ocesses in the cell cycle. There is no need to defend the value of models organising thinking about particular problems but it must be em-asised that the models in this essay are based on a relatively limited ount of information. The study of the bacterial cell cycle, as an

integrated process, is only just beginning and we cannot expect mode made at this stage to be more than tentative. However it seems alread clear that the study of the cell cycle as a whole is revealing the existen of novel molecular control systems.

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